In the cascade of reactions leading to prostaglandins, a peroxidatic enzyme reduces the hydroperoxides formed by oxygenation of unsaturated fatty acids. This manuscript describes the enzymology of a peroxidase which utilizes prostaglandin G₂, 15-hydroperoxyprostaglandin E₂, 15-hydroperoxy-5,8,11,13-eicosatetraenoic acid, 12-hydroperoxy-5,8,10,14-eicosatetraenoic acid, or H₂O₂ as substrate. A variety of radical-scavenging, reducing co-substrates stimulated the reaction and were oxidized in conjunction with hydroperoxide reduction. Enzyme-dependent co-substrate oxidation was studied qualitatively with phenol and quantitatively with cis-5-fluoro-2-methyl-1-[(p-(methylsulfinyl)benzylidene)]indene-3-acetic acid (sulindac sulfide). Molar equivalence between sulindac sulfide oxidation and 15-hydroperoxyprostaglandin E₂ reduction was observed under certain conditions.

The kinetics of the peroxidase reaction indicated substrate-dependent, irreversible self-deactivation. Furthermore, EPR measurements demonstrated the generation of radicals during enzymatic hydroperoxide reduction. These signals were quenched by each stimulatory co-substrate. Consequently, autodeactivation of peroxidase activity is interpreted as oxidative attack on the enzyme, a mechanism consistent with co-substrate oxidation by a highly reactive species. Stimulation of the peroxidase would, therefore, reflect the protective effect of radical-scavenging, reducing agents, leading to increased substrate turnovers prior to destru ctive attack. In vivo, this system could provide oxidizing equivalents either for essential metabolic processes or a self-destruct mechanism in response to excessive hydroperoxide biosynthesis.

A number of enzymatic reactions are involved in the oxygenation of arachidonic acid. Two such mammalian enzymes of importance are prostaglandin cyclooxygenase (endoperoxide synthetase) (1) and the lipoxygenases (2). The former catalyzes a double dioxygenase reaction which leads to prostaglandin G₂ (PGG₂)¹ and PGH₂ (3, 4). These endoperoxides are the precursors of the large variety of physiologically significant prostaglandins and thromboxanes. Subsequent to oxygenation of arachidonic acid by the cyclooxygenase, the 15-hydroperoxy group on PGG₂ is reduced to the hydroxyl on PGH₂ by a peroxidase (5–8). This peroxidatic activity, which copurifies with the oxygenase (9, 10), is stimulated by phenols, tryptophan, and a variety of other agents (6, 11–13). Each of these stimulators is susceptible to oxidation and in certain cases this oxidation has been demonstrated (5).

Many of the enzymes involved in prostaglandin formation including endoperoxide synthetase (9, 10, 14), endoperoxide isomerase (15), thromboxane synthetase (16), and mammalian lipoxygenase (17) have been purified to varying degrees. The ovine cyclooxygenase is a glycoprotein with a molecular weight of 126,000 (10), whereas the bovine enzyme has a subunit weight of 85,000 but a total molecular weight of 300,000 (9). Nevertheless, in both preparations, heme-containing materials and other cofactors are required for enzyme activity.

In response to substrate oxygenation (18), the cyclooxygenase undergoes natural autodeactivation, which has been interpreted as irreversible attack on the enzyme by a highly, yet indiscriminately reactive oxidant released during hydroperoxide reduction (6). During the metabolism of PGG₂ by seminal vesicle microsomes, formation of a radical was detected by EPR measurements (6). It was, therefore, postulated that the oxidant was either a radical itself or was capable of inducing radical formation. Hence, attack by this oxidant may have been the process which caused irreversible enzyme deactivation, and radical-scavenging reducing agents would have stimulated the reaction by protecting the enzyme. This concept has led to the detailed studies of the peroxidase described in this paper.

The effects of these stimulatory cofactors have also been examined in cell culture systems. With mouse 3T3 fibroblasts and human platelets, cressols and phenol depressed prostaglandin biosynthesis (19). On the other hand, prostaglandin formation was stimulated by benzo(a)pyrene (a stimulator of prostaglandin cyclooxygenase (5)) in canine kidney cells (20, 21) or by isoproterenol in rabbit kidney medullary cells (22). Furthermore, the anti-inflammatory activity of phenols has been postulated to depend upon their ability to scavenge oxidizing species (23).

This communication describes the enzymology of a microsomal peroxidase which preferentially reduces prostaglandin and other lipid hydroperoxides. The kinetics, radical generating characteristics, requirements for stimulatory cofactors, and the metabolism of these reducing co-substrates are also discussed in terms of a proposed mechanism for the enzyme.

¹ The abbreviations used are: PGG₂, PGH₂, PGF₂α, PGF₂β, PGF₃α, prostaglandins G₂, H₂, F₂α, F₂β, and E₂; 15-HPEE, 15-hydroperoxy-PGÉ; 15-HPETE, 15-hydroperoxy-5,8,11,13-eicosatetraenoic acid; 15-HETE, 15-hydroxy-5,8,11,13-eicosatetraenoic acid; 12-HPETE, 12-hydroperoxy-5,8,10,14-eicosatetraenoic acid; 12-HETE, 12-hydroxy-5,8,10,14-eicosatetraenoic acid; sulindac, cis-5-fluoro-2-methyl-1-[(p-(methylsulfinyl)benzylidene)]indene-3-acetic acid; sulindac sulfide, cis-0-fluoro-2-methyl-1-[(p-methylthio)benzylidene]indene-3-acetic acid.
EXPERIMENTAL PROCEDURES

Materials

Ram seminal vesicles, obtained from a local slaughterhouse, were stored at -70°C and used within 6 months. Soybean lipoxidase (type I) was purchased from Sigma Chemical Co. Arachidonic acid was from P-L Biochemicals, Inc., and [1-14C]arachidonic acid (58 mCi/mmole) was from Dhom Products, Ltd. The [1-14C]phenol (10.7 mCi/mmole), [1-14C]prostaglandin E, (40 mCi/mmole), 5,6-Hprostaglandin E2 (90.85 Ci/mmole), and Liquid phenol were purchased from New England Nuclear. Prostaglandin E2 was obtained from Ono Pharmaceutical Co., Ltd., and ascorbic acid was from ICN Pharmaceuticals, Inc. 12 Hydroxy-5,8,10,14-eicosatetraenoic acid (12-HPETE) and 12-hydroxy-5,8,10,14-eicosatetraenoic acid (12-HETE) were gifts from Dr. E. A. Hall of Merck & Co. Both were prepared from human platelets according to the procedure of Nutteren (17). Nonradioactive 15-HPETE and 15-HPG were obtained from standard suppliers.

Methods

Preparation of Microsomal Tissue—A suspension of microsomal tissue containing the enzymes of interest was prepared from ram seminal vesicles by homogenization and differential centrifugation as described previously (6) then stored at -70°C under nitrogen. Protein content of the microsomal suspension was determined in 5 mM sodium citric acid. The products and remaining starting materials were extracted into ether (>95% recovery) which was precooled to -70°C, then backwashed with water at 0°C, and evaporated to dryness at -35°C. After redissolving in a small amount of dry acetone, extracts were chromatographed at 25°C on Whatman SG-81 silica-impregnated paper using chloroform/ethyl acetate/acetic acid (50:50:1) as eluting solvent. Radioactive components on the chromatograms were located and quantitated with the radiochromatogram scanner. Radiolabeled spots were identified by comparison with nonradioactive standards, seen as uv-absorbing zones.

Electron Paramagnetic Resonance Measurements.—The EPR spectra were recorded on a Varian E109-E spectrometer equipped with a variable temperature accessory. Reaction mixtures were flushed with nitrogen and placed in the cylindrical EPR tube by adding starting materials, peroxide substrate, and a nonradioactive arachidonic acid solution (28) and Tappel (29) were used to prepare 15-HPETE. A solvent solution of 2.45 mCi of [1-14C]arachidonic acid (58 Ci/mmol) and 20 mg of arachidonic acid. After stirring for 60 s, the reaction was quenched with 0.3 ml of 1.56 mCi/mmol of methanol was added to 53 mg of nonradioactive arachidonic acid in 12.5 mL of 100 mM potassium phosphate buffer (pH 7.0) at 25°C. This mixture was stirred for 60 s and then acidified with 3 mL of 2 M citric acid. Both the products and the remaining arachidonic acid were immediately extracted into redistilled ether ( precooled to 0°C). The ether was backwashed with cold water, reduced in volume by evaporation, and chromatographed at -20°C on a 12-cm-wide sheet of Whatman SG-81 silica-impregnated paper using ether/hexane/acetate (50:50:50) eluting solvent. All other chemicals and materials were obtained from standard suppliers.

Preparation of [1-14C]Prostaglandin G,—A solution containing 25 mg of [1-14C]PGH, (1.45 mCi/mmol) and 1.0 mg of arachidonic acid in 1.56 mL of methanol was added to 53 mg of nonradioactive microsomal protein in 12.5 mL of 100 mM potassium phosphate buffer (pH 7.0) at 25°C. This mixture was stirred for 60 s and then acidified with 3 mL of 2 M citric acid. Both the products and the remaining arachidonic acid were immediately extracted into redistilled ether (precooled to 0°C). The ether was backwashed with cold water, reduced in volume by evaporation, and chromatographed at -20°C on a 12-cm-wide sheet of Whatman SG-81 silica-impregnated paper using ether/hexane/acetate (50:50:50) eluting solvent. All other chemicals and materials were obtained from standard suppliers.

Preparation of 1-[14C]Prostaglandin H,—To a mixture containing 65 mg of microsomal protein and 250 mM 2-(aminomethyl)-4-(1,1-dimethylthyl)-6-isodophenol were supplied by Merck & Co., Inc., cis-5-Fluoro-2-methyl-1-[r-methylthio]benzylidine)-inden-3-acetic acid (sulindac sulide; see Fig. 3 for structure), [methylene-3H]sulindac sulide at 49.8 mCi/mmole, cis-5-fluoro-2-methyl-1-[r-methylsulfinyl]benzylidine)-inden-3-acetic acid (sulindac; see Fig. 3 for structure) and [methylene-3H]sulindac at 28.8 mCi/mmole were also provided by Merck & Co., Inc. Silica Gel GF thin layer plates were supplied by Analtech, Inc., Silica Gel OF thin layer plates by New England Nuclear, and Whatman SG-81 silica-impregnated paper was from P-L Biochemicals, Inc., and [1-14C]arachidonic acid (58 mCi/mmol) was from Dhom Products, Ltd. The [1-14C]phenol (10.7 mCi/mmole), [1-14C]prostaglandin E, (40 mCi/mmole), 5,6-Hprostaglandin E2 (90.85 Ci/mmole), and Liquid phenol were purchased from New England Nuclear. Prostaglandin E2 was obtained from Ono Pharmaceutical Co., Ltd., and ascorbic acid was from ICN Pharmaceuticals, Inc. cis-5-Fluoro-2-methyl-1-[r-methylthio]benzylidine)-inden-3-acetic acid. Products and remaining starting materials were extracted into ether (>95% recovery) which was precooled to -70°C, then backwashed with water at 0°C, and evaporated to dryness at -35°C. After redissolving in a small amount of dry acetone, extracts were chromatographed at 25°C on Whatman SG-81 silica-impregnated paper using chloroform/ethyl acetate/acetate (50:50:1) as eluting solvent. Radioactive components on the chromatograms were located and quantitated with the radiochromatogram scanner. Radioactive spots were identified by comparison with nonradioactive standards, seen as uv-absorbing zones.

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acetic acid (25:75:1). The yield was 60% of 15-HPETE at 18.6 μCi/mm. Using a similar procedure, 15-HPETE was also prepared at 1.3 μCi/μmol.

Preparation of [1,14C]Hydroxy-5,8,11,13-eicosatetraenoic Acid (15 HETE)—The method of Hamberg and Samuelsson (28) was used to reduce 15-HPETE to 15-HETE at 18.6 μCi/mm. Sodium borohydride (46 μg) was added to 11.7 μg of 15-[1,14C]HPETE in 2.5 ml of dry methanol at 0°C. After incubating at 0°C for 20 min and 25°C for 40 min, the mixture was diluted with 40 ml of water and acidified with dilute hydrochloric acid. The ether extract was worked up and chromatographed as described above for PGG2 using ether/hexane/acetic acid (85:15:0.1) as developing solvent. The yield of 15-[1,14C]HETE was 88%.

RESULTS

Hydroperoxide Metabolism—Metabolism of PGG2, 15-hydroxy-PGE1 (15-HPE1), and 15-hydroperoxy-5,8,11,13-eicosatetraenoic acid (15-HPETE) by an enzyme in microsomes prepared from ram seminal vesicles has been studied using 14C-labeled substrates. The upper traces of Fig. 1 are radiochromatograms of the products and unreacted substrate in ether extracts from the three reaction mixtures. The PGG2 was reduced to PCH2, the 15-HPE1 to PGE1, and the 15-HPETE to two products, one of which was 15-hydroxy-5,8,11,13-eicosatetraenoic acid (15-HETE). In each case, comparable incubations showed less than 5% of the enzymatic reaction when conducted either with denatured enzyme (up to 90°C for 30 min) or in the absence of enzyme.

Panel A displays the metabolism of PGG2, the purity of which was established by chromatographic characterization against a standard sample (6). In the absence of additive, 11% of the PGG2 was converted to PCH2. Both materials produced positive Fe(SCN)2 peroxide tests (26), as did 15-HPE1 and 15-HPETE. Although reaction conditions leading to only minimal PGG2 reduction are displayed here in order to demonstrate the dramatic stimulatory effect of phenol (lower trace of Panel A), higher protein levels elicited greater yields of PGH2. Furthermore, with higher protein levels or longer incubation times, products of PGH2 metabolism (6-keto-PGF1α, PGE2, PGF2α) were observed near the origin but were not resolved in this chromatography system.

The enzymatic reduction of 15-HPETE to PGE1 is demonstrated in Fig. 1B. As in Panel A, these peaks were characterized by chromatographic comparison against standards. In the reaction with no additive, about 40% of the 15-HPE1 was converted to PGE1. The last traces (Panel C) show the metabolism of 15-HPETE. The peaks designated 15-HPETE and 15-HETE co-chromatographed with samples prepared and characterized as described under “Methods.” In the upper trace, 32% of the 15-HPETE was converted to 15-HETE. However, a significant quantity of another more polar product was also formed. It was established that this second product was not a peroxide. Since no 15-HETE metabolism was detected under these conditions, it evolved enzymatically from 15-HPETE rather than from secondary breakdown of 15-HETE.

By conducting all three reactions under identical conditions for 30 s at 24 μM substrate and 1.2 mg of protein/mL, the relative activities of the hydroperoxides have been established. Based on a value of 100% for PGG2, 15-HPE1, and 15-HPETE reacted 77% and 105%, respectively.

Stimulation of the Peroxidase by Co-substrates—The effects of several materials were examined on the hydroperoxide reduction reactions shown in Fig. 1. For example, the outcome of incubations containing phenol are depicted in the three bottom traces of that figure. Increased conversion does not necessarily reflect a change in initial rate, since, due to deactivation of the enzyme (see Fig. 2), reactions were complete within the 30-s incubation period. The conversion of PGG2 to PGH2 was increased about 7-fold by 500 μM phenol. Likewise, 15-HPE1 reduction to PGE1 was increased about 4-fold by 50 μM phenol. On the other hand, 500 μM phenol altered the distribution as well as the amount of products from 15-HPETE metabolism. Although the utilization of 15-HPETE increased as expected (from 60 to 100%), the amount of polar product also decreased from 15 to 5%. Hence, 15-HETE was also generated at the expense of polar material, apparently

![Fig. 1. Metabolism of hydroperoxides. The reaction conditions, work-up procedures, and techniques for analyzing radioactive products were described under "Methods." In each trace, radioactivity is plotted vertically versus distance along the chromatogram. A, [1-14C]PGG2 (24 μg, 0.03 μCi) was incubated with 0.05 mg of microsomal protein for 30 s; B, 15-[1-14C]HPETE (71 μM, 0.024 μCi) was incubated with 0.5 mg of protein for 60 s; C, 15-[1-14C]HPETE (112 μM, 0.03 μCi) was incubated with 0.5 mg of microsomal protein for 60 s. In each panel, the dotted lines represent the location of chromatographic standards detected by mass assay. The upper traces represent reaction with no additive while the lower curves depict the same reactions in the presence of phenol.](http://www.jbc.org/)


because its formation predominated kinetically.

Listed in Table I is a series of materials whose influences on these enzymatic reactions have been established in a fashion similar to phenol. The effect of each co-substrate was concentration-dependent. However, the concentration differences listed in the table preclude quantitative comparison, a comparison which would not contribute significantly to the present discussion anyway. The percentage changes listed in the last three columns were based on extent of reaction and were calculated relative to controls without any additive. Under these conditions, none of the hydroperoxides were susceptible to nonenzymatic reduction. Those compounds listed in the top half of the table, phenol, aminopyrine, diethylthiocarbamate, promethazine, sulindac sulfide, lipoic acid, methional, and tryptophan, stimulated the reduction of each hydroperoxide. On the other hand, those in the bottom half of Table I, anisole, salicylic acid, reduced glutathione, methionine, sulindac, and indomethacin had either no significant effect or were mild inhibitors. Hence, the action of a given compound was the same for each hydroperoxide. Furthermore, in every instance, PGG2 reduction was more susceptible to stimulation than 15-HPE, or 15-HPETE. The response to stimulators was also examined under identical conditions using 25 μM concentrations of PGG2 and 15-HPE, with 0.014 mg of protein. Under these conditions, 500 μM phenol was 6 times more effective a stimulator of PGG2 metabolism than that for 15-HPE. The differential in stimulation extended from 50 to 500 μM phenol. Kinetics of PGG2, 15-HPE, and 15-HPETE Reduction—The kinetics of the microsome-catalyzed reactions of PGG2, 15-HPE, and 15-HPETE were studied under the conditions described in Fig. 2, where the percentage of substrate remaining is plotted against reaction time. In order to simplify the interpretations, the PGG2 and 15-HPE1 reactions were followed in the presence of 500 μM phenol. Under these conditions, PGG2 was the sole product of PGG2 reduction and only 15-HETE resulted from 15-HPETE. The reaction of 15-HPE1 exclusively to PGE1 was performed without phenol. As depicted in Fig. 2, addition of PGG2 to the buffer containing microsomes provoked a burst of rapid metabolism culminating in about 30% conversion to PGH2 during the first 15 s. Within this period, the reaction terminated and only an additional 5% was reduced in the remaining 75 s. However, a fresh aliquot of protein, added to the inactive reaction mixture at Point A (30 s), catalyzed further metabolism, consuming 20% of the substrate, again in about 15 s. The reaction then entered another period of inactivity.

The kinetic data with 15-HPE and 15-HPETE were qualitatively identical with the PGG2 case. About 40% of the 15-HPE was converted to PGE1 in about 15 s. No significant reaction occurred in the ensuing 165 s, even following a second addition of 15-HPE at Point A (open triangle). In contrast, a second protein addition at this point reduced another 30% of the hydroperoxide. 15-HPETE metabolism also proceeded rapidly for the first 15 s, giving about 25% conversion to 15-HETE. Although a period of inactivity, likewise, succeeded this initial reaction and a second 15-HPETE addition at Point A had no effect (open square), the second protein addition elicited about 20% metabolism. The rapid secondary reaction was followed by the plateau of inactivity.

Enzyme-dependent cessation of reaction was due neither to product inhibition since addition of fresh enzyme evoked further conversion nor to substrate depletion since the reaction ceased in the presence of excess substrate. The reactions with PGG2 were shorter in duration to avoid nonenzymatic degradation which occurred due to its innate instability in

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**Table I**

Stimulation of hydroperoxide metabolism by co-substrates

The procedures for the incubations and subsequent workup were described in the legend to Fig. 1 and under "Methods." Reactions with PGG2 contained 24 μM [1-14C]PGG2 and 0.05 mg of protein whereas those with 15-HPE, had 88 μM 15-[1-14C]HPE, and 0.15 mg of protein, and those with 15-HPETE had 118 μM 15-[1-14C]HPETE and 0.05 mg of protein. The values represent the average of at least three independent incubations.

| Co-substrate  | Co-substrate concentration | Percentage change | PGG2 | 15-HPE | 15-HPETE |
|---------------|---------------------------|-------------------|------|--------|----------|
| Phenol        | 2000                      |                   | -10  | -5     | -27      |
| Aminopyrine   | 2000                      |                   | 10   | +5     | +9       |
| Diethyldithiocarbamate | 2000             |                   | 200  | +8     | +18      |
| Promethazine  | 2000                      |                   | 100  | +252   | +264     |
| Sulindac sulfide | 2000                  |                   | 100  | +252   | +257     |
| Lipoic acid   | 2000                      |                   | 100  | +465   | +228     |
| Methional     | 2000                      |                   | 200  | +345   | +111     |
| Tryptophan    | 2000                      |                   | 500  | +150   | +57      |
| Anisole       | 2000                      |                   | -100 | 0      | -27      |
| Salicylic acid| 2000                      |                   | 2000 | +10    | +5       |
| Glutathione (red) | 500                |                   | 500  | -10    | -7       |
| Methionine    | 2000                      |                   | 2000 | +10    | +8       |
| Sulindac      | 2000                      |                   | 500  | 0      | -31      |

*Plus indicates stimulation and minus indicates inhibition of the reaction relative to control incubations containing no co-substrate.

ND, not determined.
water. This difficulty did not arise within 3 min for 15-HPE, or 15-HPETE.

**Formation of Radicals during Hydroperoxide Reduction**

The enzymatic generation of an EPR signal was monitored following the addition of several hydroperoxides or their reduction products to 300 μl of microsomal suspension. Table II lists these substrates along with their concentrations. As described previously (6), the enzymatic metabolism of arachidonic acid (actually the PGG₂ formed during its oxygenation) produced an indomethacin-sensitive EPR absorption. This signal, whose peak-to-peak intensity has been arbitrarily designated 100, was depressed 93% by conducting the reaction in the presence of 500 μM phenol. In contrast, the first non-hydroperoxide in the prostaglandin-forming cascade, PGH₂, gave virtually no signal even at 500 μM. Likewise, metabolism of 15-HPE, and 15-HPETE produced signals of 63 and 84, respectively, whereas their reduction products PGE₁ and 15-HETE gave none even at 1000 μM. Phenol also depressed the signals from these hydroperoxides greater than 90%.

The primary product of platelet lipooxygenase (2), 12-HETE, generated a signal 94% as intense as that for arachidonic acid; 95% depression occurred with 500 μM phenol. Hydrogen peroxide, the only non lipid hydroperoxide studied, also formed a phenol-sensitive signal about 60% as intense as arachidonic acid. On the other hand, ascaridole, another endoperoxide like PGH₂, gave no EPR signal.

In each instance, the signals had a single peak, a line width around 25 G and no discernible hyperfine structure. Each value is the average of at least three trials. Control incubations with either enzyme alone, substrate alone, or denatured enzyme (75°C for 15 min) and substrate showed no signals.

In addition to phenol, the other co-substrates listed in Table I were tested as quenchers of the EPR signal. All those materials which stimulated hydroperoxide reduction, significantly quenched the EPR absorption with each hydroperoxide. In contrast, those materials which did not stimulate did not depress the signals.

**Peroxidase-catalyzed Oxidation of Phenol and Sulindac Sulfide**—The metabolism of phenol and of sulindac sulfide has been studied to determine whether stimulation of hydroperoxide reduction was coincident with oxidation of co-substrate. The products of [³⁵S]phenol metabolism which were generated in response to incubation with microsomal protein and arachidonic acid are shown in Fig. 3A. Of the several peaks on this radiochromatogram, phenol migrated most rapidly. The material at the origin has been identified as a polymer; it would not traverse standard gas chromatography columns and could not be volatilized in the mass spectrometer chamber. Furthermore, portions of this product adhered tightly to microsomal protein even following heat denaturation or perchloric acid treatment. Consequently, much of the radioactivity (>50%) was not extracted into ether.

Neither the phenol nor the arachidonic acid reactions occurred with denatured enzyme (75°C for 15 min) and phenol metabolism did not proceed with either enzyme alone or arachidonic acid alone. In addition, preincubation of the enzyme for 2 min with 5 μM indomethacin eliminated both arachidonic acid and phenol reactions. In accord with this well documented preincubation requirement for effective inhibition (11), failure to preincubate allowed both reactions to proceed normally. 15-HPE, also elicited the same phenol reaction as arachidonic acid.

Due to the binding and polymeric nature of the principle phenol product, the identities of the other peaks were not determined, and another stimulator of hydroperoxide reduction, sulindac sulfide, was selected for quantitative studies (structure above the small peak in Fig. 3B). This co-substrate was available in both tritiated and nonradioactive forms and,
as indicated in Table I, was a potent stimulator of the peroxidase. The radiochromatogram of the reaction mixture from the incubation of \[^3H\]sulindac sulfide with 15-HPEI in the presence of microsomal protein is shown in Fig. 3B. The sulfide was converted exclusively to sulindac, its analogous sulfoxide (structure adjacent to the larger peak). This product was identified by comparison with chemically prepared sulindac (31) in several chromatography systems and by mass spectral analysis on an LKB 9000 using direct probe insert of the solid (major peaks at 356 (mass ion), 341, and 233). The same enzymatic reaction was elicited by PGG2 and 15-HPETE. Since sulindac sulfide inhibits arachidonic acid oxygenation (ID50 = 0.1 \mu M), it was not possible to study arachidonic acid-induced oxidation and consequent indomethacin inhibition. Nevertheless, oxidation did not occur with either 15-HPEI alone, enzyme alone, or 15-HPEI with heat-denatured enzyme.

The similar kinetic patterns for the enzymatic reaction of 15-HPEI and for sulindac sulfide are shown in Fig. 4, with the per cent of unreacted radioactive substrate plotted against time. Tandem sets of incubations were performed with tritiated sulindac sulfide and nonradioactive 15-HPEI in one set and 15-[14C]HPEI and nonradioactive sulindac sulfide in the other. Under these conditions, the kinetics of 15-HPEI reduction were identical with those in Fig. 2, except that more enzyme turnovers preceded self-deactivation. Following the rapid metabolism of both components and the subsequent plateau of inactivity, a second enzyme addition at Point A elicited secondary 15-HPEI reduction and this precipitated further sulindac sulfide oxidation.

**Fig. 4.** Kinetics of 15-HPEI and sulindac sulfide reactions. The reaction conditions, work-up procedures, and detection techniques were described under "Methods." \(\bullet\), microsomal protein (0.15 mg) was incubated with 150 \mu M 15-[14C]HPEI (0.054 \mu Ci) and 100 \mu M sulindac sulfide. At Point A, either another aliquot of 15-HPEI (open circle), nothing (closed circle at 57%) or a second identical aliquot of enzyme (closed circles) was added. \(\bigtriangleup\), microsomal protein (0.15 mg) was incubated with 100 \mu M \[^3H\]sulindac sulfide (0.02 \mu Ci) and 150 \mu M 15-HPEI. At Point A, either another aliquot of 15-HPEI (open triangle), nothing (closed triangle at 42%) or a second aliquot of 0.15 mg of protein (closed triangles) was added.

**Discussion**

A natural and irreversible substrate-dependent deactivation process occurs with prostaglandin cyclooxygenase (endorpectase synthetase) (18), and a mechanism has been suggested which may explain this phenomenon (6). The proposed destructive event involved indirect attack on the oxygenase by oxidants released during a subsequent peroxidase step. This communication describes the enzymology of this microsomal peroxidase activity in order to elucidate more fully the peroxidase-mediated hydroperoxide reduction reaction and the possible effects of the resulting oxidizing equivalents on the rate, amount and direction of prostaglandin biosynthesis.

During prostaglandin formation, peroxidase activity is required to reduce the 15-hydroxyl group on PGG2 or 15-HPEI to the hydroxyl moiety present on subsequent prostaglandins. Due to the mandatory cofactor requirements of the purified cyclooxygenase (9, 10) and the consequent implications to both EPR experiments and quantitative studies of co-substrate oxidation, a microsomal preparation of the enzymes

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**Table III**

Quantitative comparison of 15-HPEI, reduction and sulindac sulfide oxidation

| 15-HPEI | Sulindac sulfide | Protein | Nanomoles metabolized |
|---------|------------------|---------|-----------------------|
| \(\mu M\) | \(\mu M\) | mg | 15-HPEI | Sulindac sulfide | Ratio |
| 150 | 100 | 0.56 | 97 | 77 | 0.79 |
| 250 | 100 | 0.56 | 103 | 70 | 0.68 |
| 500 | 100 | 0.56 | 85 | 51 | 0.60 |
| 150 | 0 | 0.56 | 25 | 25 | 1.00 |
| 150 | 100 | 0.56 | 56 | 29 | 0.52 |
| 150 | 150 | 0.56 | 97 | 77 | 0.79 |
| 150 | 150 | 0.56 | 132 | 125 | 0.95 |
| 150 | 100 | 0.20 | 56 | 56 | 1.00 |
| 150 | 100 | 0.56 | 97 | 77 | 0.79 |
| 150 | 100 | 1.11 | 128 | 88 | 0.69 |

* The molar ratio of 15-HPEI, reduced/sulindac sulfide oxidized.

Quantitative Evaluation of Co-substrate Oxidation—Since single products were formed during both 15-HPEI reduction and sulindac sulfide oxidation, this simple set of reactions was ideally suited for quantitative studies of the peroxidase. Consequently, two sets of tandem incubations with 15-[14C]HPEI, \[^3H\]sulindac sulfide, and their nonradioactive counterparts were conducted as described above. Following the work-up, greater than 95% of the radioactive component was recovered in each case.

Shown in Table III is a quantitative comparison of 15-HPEI reduction and sulindac sulfide oxidation as oxidant, reductant, or enzyme was varied. Each value represents the average of at least three trials. The molar ratios of sulindac sulfide oxidized/15-HPEI reduced are listed in the last column. For example, a ratio of 0.5 would indicate that 1 molecule of sulindac sulfide was oxidized for every two 15-HPEI molecules reduced.

As the 15-HPEI concentration was increased from 150 to 500 \mu M at 100 \mu M sulindac sulfide and 0.56 mg of protein, the molar ratio decreased from 0.79 to 0.60. On the other hand, as sulindac sulfide increased from 50 to 150 \mu M at constant 15-HPEI and protein, the ratio increased from 0.52 to 0.95. Finally, as protein level was increased from 0.20 to 1.11 mg, the ratio decreased from 1.00 to 0.69.
was used throughout the present experiments. Studies into the relative substrate selectivity of this microsomal enzyme among PGG₂, 15-HPETE, and 15-HPE₁, showed the latter to be 20% less reactive, an order of reactivity also reflected by the EPR measurements. Furthermore, the EPR signal from hydrogen peroxide was only about 60% of the control. This order of activity among the peroxides may exemplify the need for a hydrophobic environment in the immediate vicinity of the hydroperoxide group, a property lacking entirely in hydroperoxide and attenuated in 15 HPE₁ by the polar groups on the adjacent ring. For purified ovine cyclooxygenase, a similar substrate specificity was observed (10); however, with the purified bovine enzyme, 15-HPE₁ was manyfold less reactive than PGG₂ (15).

The effects of several compounds with dramatically different structures were examined on hydroperoxide reduction. The similarity in response despite the disparity in structure strongly suggests that these substances do not stimulate a specific site on the enzyme. This stimulation occurred with no detectable effect on isomerization, an observation in accord with previous studies using arachidonic acid (32) or PGG₂ (13). On the other hand, the absence of activity by several other compounds suggests some selectivity among stimulators, perhaps based on reduction potential. The inactivity of reduced glutathione is somewhat unexpected since it is oxidizable.

Although some materials stimulated and others did not, the action of each compound tested was the same with either PGG₂, 15-HPE₁, or 15-HPETE as substrate. The reduction reactions of 15 HPE₁ and 15 HPETE were augmented almost equally in each instance; however, PGG₂ was significantly more sensitive to stimulation. The uniform action of each co-substrate with all three hydroperoxides suggests that the same enzyme rather than a series of different, yet specific, peroxidases catalyzes the three reduction reactions. As further evidence that the same enzyme catalyzed all three reactions, the kinetic patterns for peroxidatic reduction of the three hydroperoxides were qualitatively identical. A lack of both hydroperoxide and co-substrate specificity is characteristic of this class of enzymes (33).

The EPR signal detected during enzymatic metabolism of arachidonic acid, 15-HPE₁, 15-HPETE, or H₂O₂ was observed previously with either arachidonic acid or PGG₂ (6). However, the present studies include the reduction products from each of the hydroperoxides in order to demonstrate conclusively that enzymatic peroxide reduction was responsible for the signal. The absence of a signal from the two endoperoxides, PGE₂ and ascaridole, further emphasizes the obligatory requirement for a hydroperoxide moiety. Furthermore, each co-substrate which augmented the peroxidase reaction depressed the EPR signal, implying that the moiety responsible for the signal may regulate peroxidase activity.

Since a product of platelet lipoxygenase, 12-HPETE (2), also generated an EPR signal, inhibition of the cyclooxygenase by nonsteroidal anti-inflammatory agents would not preclude in vivo radical formation by this peroxidase. An appropriate hydroperoxide substrate would still be formed from arachidonic acid by the lipoxygenase and these agents do not have an effect on either lipoxygenase or peroxidase reactions. In fact, the capacity of this enzyme to produce radicals from nonprostaglandin hydroperoxides may relate to the pathology of these other hydroperoxy fatty acids (34).

Based on these observations, we conclude that the auto-deactivation of this enzyme indicated by its kinetics with all three hydroperoxide substrates may occur by a mechanism similar to that postulated for the cyclooxygenase (6), self-inflicted oxidative attack. The deactivation is considered irreversible because neither standing for 20 min nor adding further hydroperoxide or phenolic substrate could reinstate any activity, whereas fresh enzyme elicited further reaction. Stimulation by co-substrates could then involve preservation of the enzyme and an increase in substrate turnovers prior to deactivation. Deactivation occurred more rapidly for the peroxidase than for the cyclooxygenase, possibly reflecting its closer proximity to the site of oxidant formation. Alternatively, the susceptibility of the enzyme itself must be considered.

If these radical-scavenging reducing agents were, indeed, acting as co-substrates during hydroperoxide reduction, they should have been metabolized in a peroxidase-dependent fashion. Of the several products formed from phenol, at least one was polymeric, an expected result of phenol oxidation (35). Although our experiments qualitatively confirmed the role of phenol as reducing co-substrate for the peroxidase, further studies were precluded due to the intractable nature of the products and another co-substrate, sulindac sulfide, was chosen for quantitative studies of this reaction (see Fig. 3 for structure). Although sulindac sulfide stimulated the peroxidase, it inhibited the addition of oxygen to arachidonic acid by the cyclooxygenase. These dual and opposite actions clearly separate the oxygenase from peroxidase activities, although they seem to reside on the same protein molecule (9, 10, 14).

The kinetic behavior of peroxidase-dependent sulindac sulfide oxidation was qualitatively identical with that for 15-HPE₁ reduction. The similarity between the unusual kinetics of these two reactions indicates that oxidation of the co-substrate was inextricably associated with peroxidase activity. However, this does not necessarily imply direct reaction between either phenol or sulindac sulfide and the enzyme. On the contrary, co-substrate oxidation could occur as a result of chemical reaction with the oxidant released during hydroperoxide reduction.

A quantitative comparison of 15-HPE₁ reduction and sulindac sulfide oxidation was also conducted. The parameter chosen for this comparison, the molar ratio of sulindac sulfide oxidized/15-HPE₁ reduced, is a measure of the efficiency of sulindac sulfide oxidation, with a ratio of 1 representing 100% utilization of the oxidant released by 15-HPE₁. As either sulindac sulfide was increased or protein was decreased, the ratio increased, implying competition between microsomal tissue and sulindac sulfide for the oxidant. This is in accord with the capacity of the oxidant to attack and deactivate enzymes. Under conditions of high sulindac sulfide or low protein, where attack on the microsomal tissue would be minimized, the molar ratio approached 1, a stoichiometric reaction of 1 mol of sulindac sulfide with each mole of 15-HPE₁. Although the capacity of each co-substrate to compete for the oxidizing species may vary depending upon its reduction potential and molecular structure, this value is in agreement with the stoichiometry found with hydroquinone and purified cyclooxygenase (10).

In summary, this paper describes a microsomal peroxidase which is intimately involved in prostaglandin biosynthesis. With broad specificity, it can utilize hydroperoxides formed by other oxygenating systems such as mammalian or plant lipooxygenases. During hydroperoxide reduction, oxidizing species which may be either radicals themselves or capable of inducing radical formation are generated. Attack on the peroxidase by these oxidants may be the event leading to its irreversible auto-deactivation. These same moieties can also oxidize reducing co-substrates which stimulate the peroxidase by dint of substituting for and, thereby, protecting the enzyme from deactivation. The highly yet indiscriminately reactive nature of this oxidant suggests that it may play a role in
cellular function, either as an intermediate in obligatory metabolic oxidations or as a destructive reaction in response to uncontrolled hydroperoxide biosynthesis.

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