Biochemistry

 Volume 18, Number 14 July 10, 1979

Peroxidatic Oxidation of Benzo[a]pyrene and Prostaglandin Biosynthesis†

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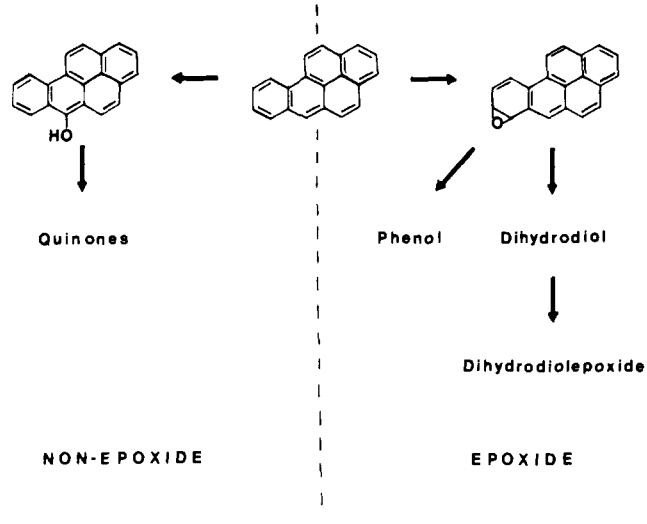
ABSTRACT: The arachidonic acid dependent oxidation of benzo[a]pyrene to a mixture of 3,6-, 1,6-, and 6,12-quinones has been studied by using enzyme preparations from sheep seminal vesicles. Maximal oxidation is observed at 100 μM benzo[a]pyrene and 150 μM arachidonic acid. The arachidonic acid dependent oxidation is peroxidatic and utilizes prostaglandin G_2 (PGG_2), generated in situ from arachidonate, as the hydroperoxide substrate. 15-Hydroperoxy-5,8,11,13-eicosatetraenoic acid is equivalent to PGG_2 as a hydroperoxide substrate, but hydrogen peroxide, cumene hydroperoxide, and *tert*-butyl hydroperoxide are much poorer substrates. Arachidonic acid dependent benzo[a]pyrene oxidation by microsomal and solubilized enzyme preparations is markedly stimulated by a variety of hemes and heme proteins. This is not due to the previously reported heme stimulation of prostaglandin biosynthesis [Yoshimoto, A., Ito, H., & Tomita, K. (1970) *J. Biochem. (Tokyo) 68*, 487–499]. Instead, the hemes function directly as peroxidases utilizing fatty acid hydroperoxides as substrates. The incubation of PGG_2 with commercial methemoglobin in the absence of any other protein gives rise to significant benzo[a]pyrene oxidation to quinones. The widespread occurrence of heme proteins in animal tissue suggests that the peroxidatic oxidation of benzo[a]pyrene will be significant in any tissue that makes appreciable concentrations of fatty acid hydroperoxides.

A substantial body of experimental evidence suggests that chemical carcinogens are electrophiles which bond covalently to cellular nucleophiles in an event fundamental to cell transformation (Miller & Miller, 1966; Miller, 1970; Heidelberger, 1975). Polycyclic aromatic hydrocarbons, an abundant class of carcinogens, are not electrophilic but are converted in vivo to reactive derivatives during oxidative metabolism (Miller, 1951; Heidelberger & Weiss, 1951). The metabolism of benzo[a]pyrene[1] in animal tissue involves a combination of oxidation and conjugation steps (Falk et al., 1962; Sims, 1967; Selkirk et al., 1976). The oxidation steps have been implicated as the key reactions in the activation sequence and have been shown to be catalyzed by the NADPH dependent mixed-function oxidases (Gelboin, 1969).

The two general pathways of oxidative metabolism of BP are shown in Scheme I. In one pathway, BP is oxygenated to unstable epoxide intermediates (Grover et al., 1972). These compounds are either enzymatically hydrated to dihydrodiols or nonenzymatically rearranged to phenols (Holder et al., 1974). In the other pathway, BP is oxidized to 6-OH-BP which nonenzymatically oxidizes in aerobic solutions to a mixture of quinones (Nagata et al., 1974; Lorentzen et al., 1975; Sullivan et al., 1978). Neither the formation nor the decomposition of 6-OH-BP is believed to involve epoxide intermediates. Intermediates in both the epoxide and the nonepoxide pathways are carcinogenic and have been proposed to be metabolically activated forms of BP (Levin et al., 1977; Slaga et al., 1977; Nagata et al., 1974). Recent studies,

Scheme I



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[1] Abbreviations used: BP, benzo[a]pyrene; 20:4, arachidonic acid; metHb, methemoglobin; metMb, metmyoglobin; 15-HPEA, 15-hydroperoxy-5,8,11,13-eicosatetraenoic acid; PGG_2, 15α-hydroperoxy-9α,11α-peroxido-5-*cis*,13-*trans*-prostadienoic acid; $PGF_{2\alpha}$, 9α,11α,-15α-trihydroxy-5-*cis*,13-*trans*-prostadienoic acid; PGH_2, 15α-hydroxy-9α,11α-peroxido-5-*cis*,13-*trans*-prostadienoic acid; EPR, electron paramagnetic resonance; HETE, 12-hydroperoxy-5,8,10,14-eicosatetraenoic acid.

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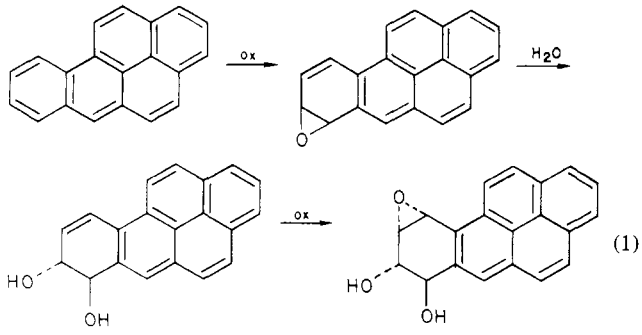
however, implicate dihydrodiol epoxides, produced as shown in eq 1, as the ultimate carcinogenic forms of BP (Borgen et



(1)

al., 1973; Sims et al., 1974; Huberman et al., 1976; Wislocki et al., 1976; Jeffrey et al., 1977; Meehan et al., 1977; Kapitulnik et al., 1978).

We have recently found that during prostaglandin biosynthesis, BP is oxidized to a mixture of quinones (Marnett et al., 1977). We have subsequently shown that prostaglandin synthetase also oxidizes 7,8-dihydroxy-7,8-dihydro-BP to a mutagenic derivative, possibly the ultimate carcinogen 7,8-dihydroxy-9,10-epoxy-7,8,9,10-tetrahydro-BP (Marnett et al., 1978). With both cooxidized substrates, these is an absolute dependence on prostaglandin synthetase and its substrate, arachidonic acid. This raises the possibility that prostaglandin synthetase acts in vivo as an alternate activating enzyme to the mixed-function oxidases in BP carcinogenesis. We have, therefore, undertaken a study of the prostaglandin synthetase dependent metabolism of BP using enzyme preparations from sheep seminal vesicles. This tissue was chosen for our initial study because of its extremely high prostaglandin biosynthetic capacity and its very low mixed-function oxidase activity (Marnett et al., 1977). We have concentrated on the metabolism of BP because our original investigations indicate that prostaglandin synthetase dependent oxidation proceeds by the nonepoxide pathway (Marnett et al., 1977). In contrast to the mixed-function oxidases, prostaglandin synthetase oxidizes BP solely by this mechanism and should, therefore, constitute a good system for the study of the biochemistry associated with this pathway.

Experimental Procedures

Materials. [7,10-^{14}C]BP (60.7 mCi/mmol) and [U-^{3}H]BP (24 Ci/mmol) were purchased from Amersham/Searle. [1-^{14}C]-20:4 was from Applied Science. Unlabeled 20:4 was kindly provided by Dr. John Paulsrud, Hoffman-La Roche. Whale skeletal muscle metMb, hematin, protoporphyrin IX, and yeast tRNA (soluble; A grade) were from Calbiochem. BP, ADP (equine muscle, Grade I), lipoxygenase, metHb (beef blood), beef liver catalase (crystalline suspension; 2× crystallized), and indomethacin were from Sigma. Cumene and *tert*-butyl hydroperoxides were from ICN. Glass fiber filters were from Whatman. Standard metabolites of BP were obtained through the National Cancer Institute Carcinogenesis Research Program. All other chemicals and solvents were from Fisher.

15-HPEA was biosynthesized from 20:4 by the action of soybean lipoxygenase (Funk et al., 1976). PGG_2 was biosynthesized from 20:4 according to published procedures (Hamberg et al., 1974). It was quantitated following reduction to $PGF_{2\alpha}$. H_2O_2, cumene hydroperoxide, and *tert*-butyl hydroperoxide were assayed by iodometric titration (Mair & Graupner, 1964). The heme content of heme-containing preparations was assayed by pyridine hemochromogen formation (Paul et al., 1953). Thymol was removed from catalase by passing the preparation through Sephadex G-25 immediately prior to use. Commercial tRNA was purified by ion-exchange chromatography (Monier et al., 1960). Protein was determined according to Lowry et al. (1951).

Enzyme Preparations. The microsomal fraction and a Tween 20 solubilized enzyme were prepared from sheep seminal vesicles as previously described (Marnett & Wilcox, 1977).

Incubation Conditions. All incubations were carried out in 0.1 M potassium phosphate (pH 7.8) at 25 °C in a total volume of 1.0 mL. Hemin chloride and protoporphyrin IX were added in buffer–ethanol, 1:1. Heme proteins were added in buffer. PGG_2 and 15-HPEA were added in acetone; indomethacin, cumene hydroperoxide, and *tert*-butyl hydroperoxide were added in ethanol. [7,10-^{14}C]BP (0.35 μCi/μmol) was added in acetone to incubations containing all additives except the substrate. Reaction was initiated by the addition of 20:4 or the indicated hydroperoxide. Incubation, extraction, and thin-layer chromatography (TLC) were as previously described (Marnett et al., 1977). Incubations performed with PGG_2 and 15-HPEA led to the extraction and chromatography of ^{14}C-labeled compounds derived from the hydroperoxide. To correct for this amount of radioactivity on the TLC plates, parallel duplicate incubations were run at each hydroperoxide concentration in the absence of BP. Hydroperoxide-derived radioactivity remained at the origin, and the amount of radioactivity determined in incubations with BP was corrected for this amount.

Nucleic acid binding experiments were performed under similar conditions. In 1.0 mL was contained tRNA (800 μg), [^{3}H]BP (68 mCi/mmol) (50 μM), and solubilized enzyme (800 μg of protein). Reaction was initiated by the addition of 20:4 (200 μM) and proceeded for 15 min at 25 °C. Each incubation was quenched by the addition of 1.0 mL of buffer-saturated phenol and vortexed. Binding was assayed by the method of King (1974).

Results

Figure 1 presents a comparison of the chromatographic profiles obtained when [^{14}C]BP is incubated with a mixed-function oxidase preparation from rat liver microsomes and a prostaglandin synthetase preparation from sheep vesicular gland microsomes. The mixed-function oxidase preparation catalyzes the formation of a number of products which cochromatograph with authentic standards of BP phenols and dihydrodiols. As discussed above both classes of compounds are formed via epoxide intermediates. In contrast, prostaglandin synthetase catalyzes the formation of BP quinones which arise exclusively by the nonepoxide pathway. The structures of these quinones are shown in Scheme II. The broad peak chromatographing between fractions 120 and 130 does not correspond to any known metabolite of BP. The fact that it is formed in boiled enzyme incubations suggests that it may be a nonenzymatic product of spurious origin. We have not attempted to identify this material.

Since the products of prostaglandin synthetase dependent BP oxidation are quinones, we are unable to use the routine fluorometric assays for BP oxidation activity. We have assayed BP oxidation by determining the amounts of radioactive products formed from [^{14}C]BP following incubation, extraction, and separation by TLC. Incubation times of 15 min were employed because previous work has shown that the oxidation is maximal after this time (Marnett et al., 1975). With microsomal and Tween 20 solubilized preparations from sheep seminal vesicles, maximal BP oxidation is observed at [BP] = 100 μM and [20:4] = 150 μM. At saturating con-

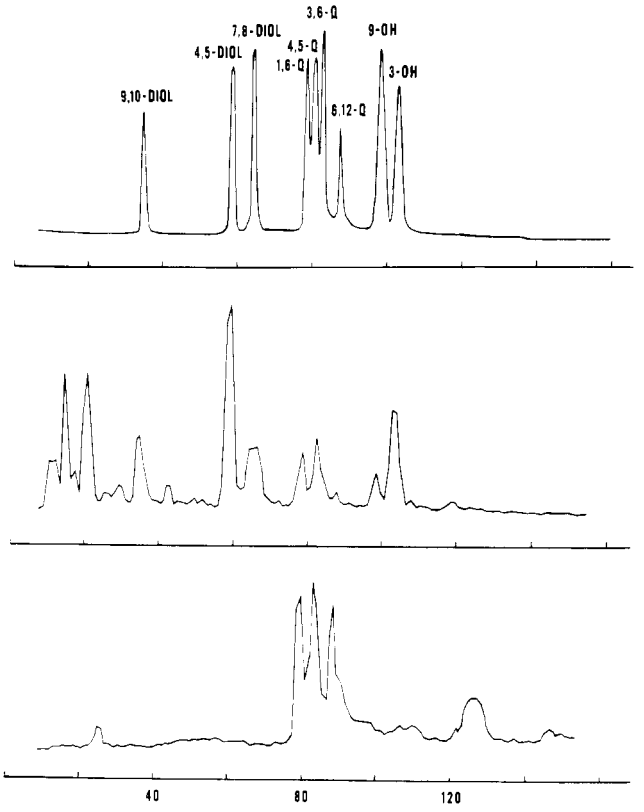


FIGURE 1: Chromatographic profiles of the products of BP oxidation by mixed-function oxidase and prostaglandin synthetase preparations. Top frame: UV profile of authentic standards of BP metabolites. The three classes of metabolites are dihydrodiols (DIOL), quinones (Q), and phenols (OH). The numbers refer to the position of substitution on the BP ring. Middle frame: radioactivity profile of the products of oxidation of [7,10-^{14}C]BP by rat liver microsomes and NADPH. Bottom frame: radioactivity profile of the products of oxidation of [7,10-^{14}C]BP by sheep vesicular gland microsomes and arachidonic acid. Chromatography was performed on a 4 × 250 mm μBondapak C_{18} reverse-phase column (Waters Associates) by using a linear gradient of 50–70% methanol–water (35-min program) at a flow rate of 1.5 mL/min.

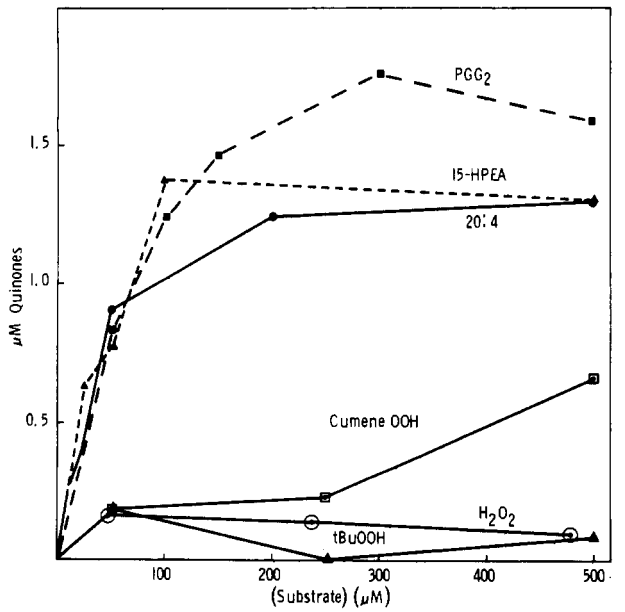


FIGURE 2: Dependence of sheep vesicular gland microsome dependent BP oxidation on the concentration of different hydroperoxides. Conditions are described under Experimental Procedures.

Scheme II

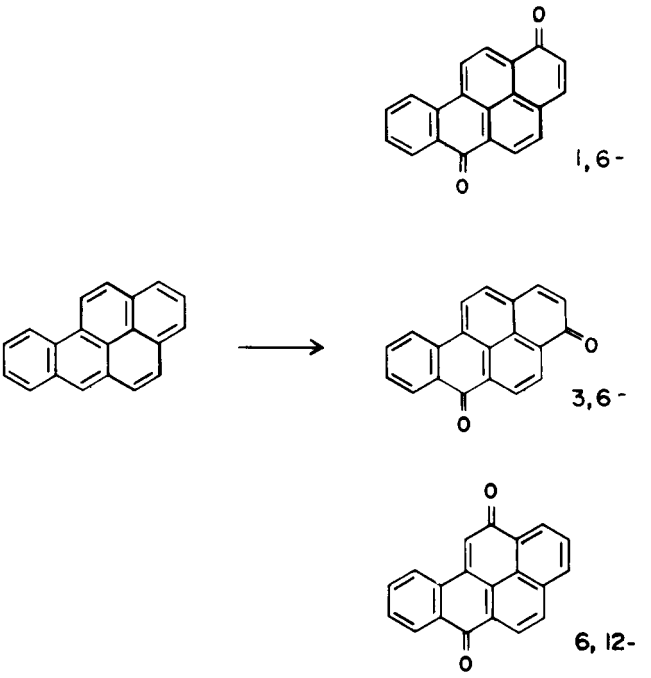


Table I: Stimulation of Prostaglandin Synthetase Dependent BP Oxidation by metHb

conditions[a]	μM quinones
10000*g* supernatant	0.8 ± 0.3
10000*g* supernatant + metHb[b]	1.3 ± 0.2
microsomal fraction	1.2 ± 0.2
microsomal fraction + metHb[b]	4.3 ± 0.2
solubilized	1.76 ± 0.04
solubilized + metHb[c]	12 ± 1

[a] All incubations were in a final volume of 1.0 mL with 125 μM [^{14}C]BP. Supernatant (10000*g*) and microsomes were added equivalent to 140 mg of tissue. Solubilized enzyme added was equivalent to 100 mg of tissue. Following a 3-min preincubation, 20:4 was added to 330 μM. Incubations were continued for 15 min at 25 °C and then quenched and assayed as described under Experimental Procedures. All values are the result of triplicate determinations. [b] [heme] = 3 μM. [c] [heme] = 3.9 μM.

centrations of 20:4, half-maximal oxidation occurs at [BP] = 20 μM. At this concentration, 3 and 30% of the added BP are oxidized when the incubations are carried out in the absence and in the presence of 1 μM metHb, respectively (vide infra).

Prostaglandin synthetase dependent BP oxidation is peroxidatic (Marnett et al., 1975). 20:4 is oxygenated to the hydroperoxy endoperoxide PGG_2, which is converted to the hydroxy endoperoxide PGH_2. The amount of BP oxidized to quinones is much less than the amount of 20:4 converted to PGH_2. Figure 2 compares the hydroperoxide specificity of the microsomal peroxidase. Included in the figure is the 20:4 concentration dependence. As expected, the concentration dependences of 20:4 and PGG_2 are very similar. It is of interest that the 20:4 derived hydroperoxides PGG_2 and 15-HPEA support significantly greater oxidation than does H_2O_2 or the organic hydroperoxides, cumene hydroperoxide and *tert*-butyl hydroperoxide.

A number of authors have reported that prostaglandin biosynthesis is markedly stimulated by hemes and heme proteins (Yoshimoto et al., 1970; Ogino et al., 1978; Hemler et al., 1978). Table I shows that prostaglandin synthetase dependent BP oxidation is stimulated by metHb. Stimulation

Table II: Heme and Heme Protein Stimulation of BP Oxidation by Tween 20 Solubilized Prostaglandin Synthetase

additive[a]	μM quinones
none–control	1.76 ± 0.04
metHb	12.5 ± 1.1
boiled metHb	10.2 ± 1.2
metMb	7.5 ± 0.6
catalase[b]	1.2 ± 0.1
metHb + catalase[c]	10.8 ± 0.6
hemin	5.20 ± 0.05
protoporphyrin IX	1.02 ± 0.05
$FeCl_3$	0.46 ± 0.01
$FeSO_4$	1.8 ± 0.5
Fe^{3+}ADP	1.5 ± 0.2
Fe^{3+}EDTA	0.5 ± 0.1

[a] All compounds were added at [Fe] or [heme] = 4 μM final concentration in 1.0-mL incubation mixtures containing 400 μL of solubilized protein and 125 μM [[^{14}C]BP] in 0.1 M KPO_4, pH 7.8. Following a 3-min preincubation, 20:4 was added at a concentration of 330 μM, and incubations continued for 15 min at 25 °C. Reported values are the result of triplicate determinations. [b] Thymol (0.1%) was removed by Sephadex G-25 chromatography. Heme content was analyzed by pyridine hemochromogen assay. [c] metHb (1 μM) + thymol-free catalase (1 μM).

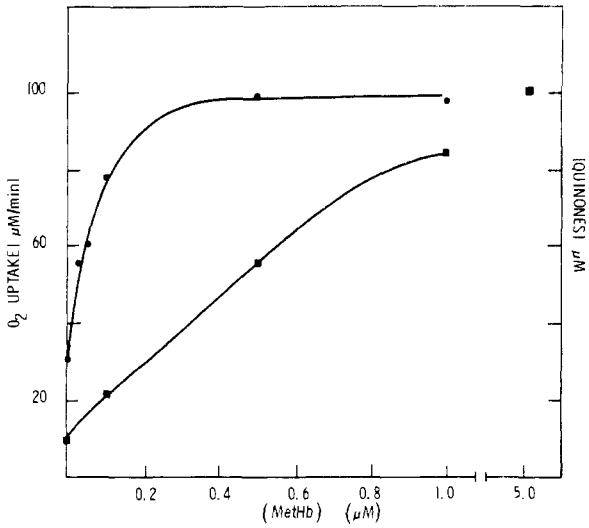


FIGURE 3: Methemoglobin stimulation of prostaglandin biosynthesis (O_2 uptake) and BP oxidation ([quinones]) by a Tween 20 solubilized enzyme preparation from sheep seminal vesicles. Conditions are described under Experimental Procedures.

is observed with the 10000*g* supernatant, the microsomal fraction, and a Tween 20 solubilized preparation. With all enzyme preparations, LC analysis demonstrates that the products of BP oxidation are identical in the presence or absence of metHb.[2] Table II demonstrates that oxidation by the solubilized enzyme is stimulated by heme proteins in a nonspecific fashion. Hemin itself stimulates appreciable oxidation but protoporphyrin IX and non-heme iron complexes do not. Catalase neither stimulates nor inhibits BP oxidation.

The compounds in Table II which stimulate BP oxidation have been reported to stimulate prostaglandin biosynthesis (Yoshimoto et al., 1970; Ogino et al., 1978). Therefore, the increased BP oxidation observed in their presence might simply be a result of the increased conversion of 20:4 to the peroxidase substrate PGG_2. To test this we have investigated the effect of metHb concentration on both prostaglandin biosynthesis and BP oxidation. Prostaglandin biosynthesis was assayed by O_2 uptake and conversion of [^{14}C]-20:4 to labeled products.

[2] John T. Johnson, *unpublished experiments.*

Table III: PGG_2 Dependent BP Oxidation by metHb under Various Conditions

conditions[a]	μM quinones	
metHb[b]	1.1 ± 0.4[c]	2.3 ± 0.3[d]
solubilized enzyme[e]	1.28 ± 0.05	1.9 ± 0.2
solubilized enzyme[e] + metHb[b]	3.0 ± 0.1	5.7 ± 0.9
boiled solubilized enzyme[e] + metHb[b]	2.3 ± 0.3	4.08 ± 0.02
Tween 20 + metHb[b]	2.06 ± 0.04	5.0 ± 0.6

[a] All incubations were carried out in 0.5-mL final volume with 125 μM [^{14}C]BP. Tween 20 incubations contained an aliquot of solubilization buffer (20 mM $NaPO_4$, pH 7.4, and 1% (w/v) Tween 20) equal to the volume of solubilized enzyme added (50 μL) to other incubations. All tubes were preincubated for 3 min at 25 °C, and then PGG_2 was added to the indicated concentrations. Incubation continued at 25 °C for 15 min. All incubations were performed in duplicate. [b] [metHb] = 1 μM. [c] [PGG_2] = 39 μM. [d] [PGG_2] = 157 μM. [e] Solubilized protein (200 μg).

Table IV: Prostaglandin Synthetase Induced Binding of BP to Yeast tRNA

conditions	tRNA binding[a]
complete system[b]	31.2 ± 0.4
complete system – 20:4	16 ± 2
complete system + indomethacin	20.3 ± 0.4
complete system (boiled enzyme)	5.4 ± 0.2
complete system (tRNA at end)	1.6 ± 0.2

[a] Picomoles per milligram of tRNA per 15 minutes. [b] The complete system consists of solubilized enzyme (800 μg of protein), [^{3}H]BP (50 μM), tRNA (800 μg), and 200 μM 20:4 in a final volume of 1.0 mL. All tubes were preincubated for 3 min at 25 °C, followed by the addition of 20:4 and 15-min incubation at 25 °C.

Figure 3 shows that metHb stimulation of prostaglandin biosynthesis is half-maximal at 80 nM and maximal at 1.5 μM heme. In contrast, a roughly linear increase in BP oxidation occurs up to 4 μM heme, and a significant increase is observed between 4 and 20 μM heme. The concentration dependence of the metHb stimulation of BP oxidation does not correlate with that of prostaglandin biosynthesis.

One possible explanation for the differential concentration dependences exhibited in Figure 3 is that metHb is acting alone or in combination with a protein in the solubilized preparation as a peroxidase. We have investigated this possibility using PGG_2 as the substrate, and the data are presented in Table III. PGG_2 and metHb do, in fact, support the peroxidatic oxidation of BP. However, the magnitude of the oxidation is less than the difference between the solubilized enzyme alone and the solubilized enzyme plus metHb. This discrepancy is due to a stimulation of the PGG_2 dependent metHb peroxidase activity by the solubilizing agent Tween 20. When this stimulation is taken into consideration, the total BP oxidation by the solubilized enzyme in the presence of metHb is approximately the sum of the oxidation by the solubilized enzyme alone and by metHb alone.

The results of the preceding experiments demonstrate that during prostaglandin biosynthesis BP undergoes peroxidatic oxidation to a mixture of quinones. An important question with regard to BP carcinogenesis is whether any of the intermediates in this peroxidatic oxidation constitute metabolically activated derivatives of BP. One way to answer this question is to include a suitable nucleophile in the incubation mixture to trap reactive electrophilic derivatives by covalent bond formation (King & Phillips, 1969; Kadlubar et al., 1977). The results of such experiments using yeast tRNA as the trapping agent are summarized in Table IV.

In the absence of added metHb 30 pmol of labeled BP derivatives is bound to tRNA. This represents approximately

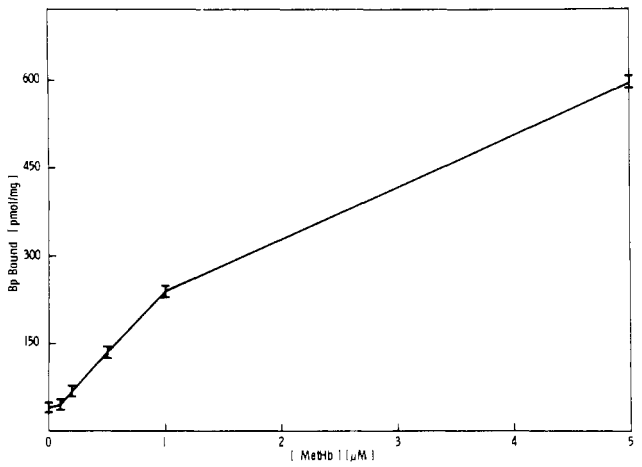


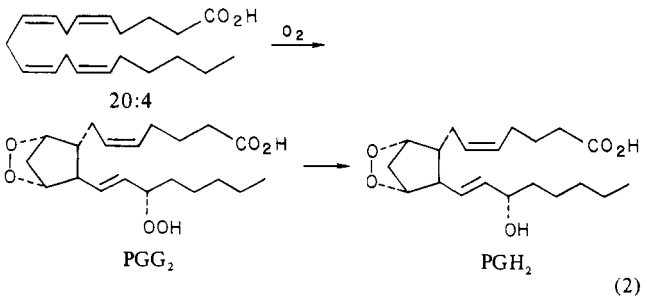
FIGURE 4: Methemoglobin stimulation of covalent attachment of radioactivity to yeast tRNA during incubation of [^{3}H]BP with a Tween 20 solubilized enzyme preparation from sheep seminal vesicles. Conditions are described under Experimental Procedures.

2% of the BP oxidized to quinones under these conditions. Control experiments indicate that the tRNA does not inhibit prostaglandin biosynthesis or BP oxidation. Reduced levels of binding occur when incubations are performed in the presence of the prostaglandin synthetase inhibitor indomethacin. Brief heating of the enzyme preparations in a boiling water bath abolishes binding. To ensure that the observed binding is not actually due to physical entrapment, a control was performed in which the tRNA was added 15 min after the initiation of reaction and 1 min before phenol extraction. Very little binding (2 pmol) was observed.

The addition of metHb to the incubation mixtures markedly stimulates the amount of labeled BP derivatives bound to tRNA (Figure 4). Up to 1 μM metHb, this is mainly the result of increased metabolism of BP since the percentage of oxidized products bound to tRNA is constant at 2%. However, at 5 μM metHb, 6% of the oxidized BP is bound to tRNA. At this Hb concentration, covalent binding to tRNA is completely inhibited by indomethacin.

Discussion

Prostaglandin synthetase (prostaglandin endoperoxide synthetase, fatty acid cyclooxygenase) appears to contain two activities (eq 2). The first of these, the cyclooxygenase,



(2)

catalyzes the oxygenation of 20:4 to the hydroperoxy endoperoxide PGG_2 (Hamberg et al., 1974; Nugteren & Hazelhof, 1973). The second, the hydroperoxidase, catalyzes the reduction of PGG_2 to the hydroxy endoperoxide PGH_2 (Miyamoto et al., 1976). It is the latter activity which appears to be responsible for the cooxygenations observed during prostaglandin biosynthesis. That is, PGG_2, generated in situ from 20:4, interacts with a microsomal peroxidase to generate an oxidizing agent which then triggers xenobiotic oxidation. In the case of BP, the products of the oxidation indicate that the enzymatically generated oxidizing agent converts BP to 6-OH-BP via the one-electron pathway (Nagata et al., 1974; Sullivan et al., 1978). 6-OH-BP then is oxidized enzymatically or nonenzymatically to a mixture of quinones (Lorentzen et al., 1975). The same mixture of quinone products has been observed following the oxidation of BP with horseradish peroxidase and H_2O_2 (Breinlich, 1964).

Hydroperoxide-dependent oxidations by cytochrome P-450 have been extensively studied in recent years (Hrycay & O'Brien, 1971; Rahimtula & O'Brien, 1975; Coon et al., 1977). The possibility exists that the cooxidations observed during prostaglandin biosynthesis arise by the interaction of PGG_2 with cytochrome P-450 in the vesicular gland microsomal fraction. Several lines of evidence argue against this possibility. First, the chromatographic profiles in Figure 1 show that BP is oxidized by vesicular gland microsomes by a different mechanism than by rat liver cytochrome P-450. This is not due to the fact that the course of the reaction varies with NADPH or hydroperoxide as initiator since hydroperoxide-dependent cytochrome P-450 oxidations give the same products by the same mechanisms as NADPH-dependent cytochrome P-450 oxidations (Rahimtula et al., 1978). Second, we are unable to detect any cytochrome P-450 in vesicular gland microsomes or the Tween 20 solubilized preparation by dithionite reduction and CO treatment (Marnett et al., 1977). Third, there is no NADPH-dependent BP oxidation in vesicular gland microsomes (Marnett et al., 1977). Fourth, incubation of PGG_2 with commercial preparations of metHb oxidizes BP to the same mixture of quinones as does vesicular gland microsomes, and the metHb surely has no cytochrome P-450 contaminant.

The oxidizing agent generated by the addition of PGG_2 to the enzyme may be either protein or substrate (PGG_2) derived. If this is a typical peroxidatic reduction, one would expect that the interaction of PGG_2 with the enzyme would form PGH_2 and a compound I derivative of the protein, two oxidizing equivalents above the resting state of the enzyme. Sequential one-electron reductions of the enzyme would complete the catalytic cycle. The oxidizing agent would, therefore, be protein derived. Alternatively, the enzyme could reduce PGG_2 by only one electron during the initial interaction, producing an alkoxy radical and an enzyme which is one oxidizing equivalent above its resting state. In this case, the oxidizing agent would be either substrate or protein derived. Such a pathway would indicate that the enzyme is not a peroxidase in the classic sense. The only investigation into the nature of the oxidizing agent produced during the peroxidatic reduction of PGG_2 is the EPR study of Egan et al. (1976). These workers have shown that the addition of PGG_2 to sheep vesicular gland microsomes results in the formation of an EPR signal which can be quenched by the addition of an oxidizable cosubstrate. Unfortunately, the lack of hyperfine structure in the EPR signal does not allow an identification of the radical to be made.

Hemes and heme proteins markedly stimulate prostaglandin synthetase dependent BP oxidation. The products formed in the presence of hemes are the same as the products formed in the absence of hemes and, therefore, also arise by the one-electron oxidation pathway. We have compared the effect of increasing concentrations of metHb on prostaglandin biosynthesis and BP oxidation by a Tween 20 solubilized enzyme preparation. It is evident from Figure 3 that there is not a correlation between the effect of metHb on these two processes. This implies that metHb and probably other hemes are acting to augment the peroxidase activity. The data in

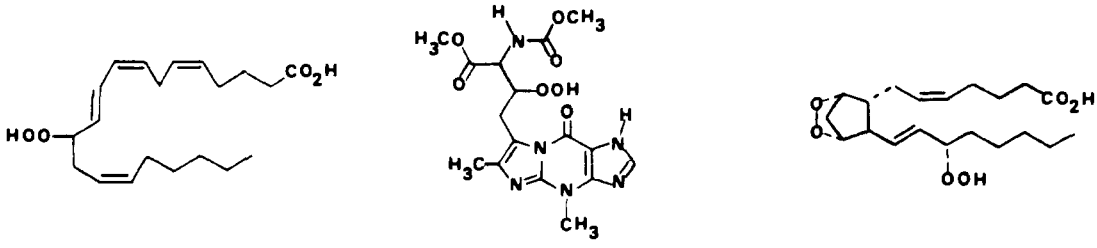


FIGURE 5: Structures of alkyl hydroperoxides isolated from animal tissue.

Table III suggest that this is due to the direct peroxidatic action of the hemes on BP utilizing PGG_2 as the hydroperoxide substrate. When account is taken of the stimulation of the metHb peroxidase activity by Tween 20, it appears that the total peroxidase activity of a Tween 20 solubilized enzyme preparation containing metHb is the sum of the peroxidase activity of the vesicular gland extract and the peroxidase activity of metHb.

The fact that a number of heme proteins catalyze the peroxidatic oxidation of BP via the one-electron pathway suggests that this oxidation might be widespread in animal tissue provided suitable hydroperoxide substrates are available. Surprisingly, the occurrence of hydroperoxides in the animal kingdom appears to be rather limited. In addition to H_2O_2 only three hydroperoxides have been isolated in the entire animal kingdom, and their structures are displayed in Figure 5.[3] It is interesting to note that two of these compounds, PGG_2 and HETE, are metabolites of arachidonic acid (Hamberg et al., 1974; Nugteren & Hazelhof, 1973; Hamberg & Samuelsson, 1974). The remaining compound, peroxy-Y-base, is a component of $tRNA^{Phe}$ and has been isolated from beef, rat, and chicken liver (Feinberg et al., 1974). The pathway of its biosynthesis is unknown. It seems likely that additional alkyl hydroperoxides will be isolated from animal sources, but at present it appears that prostaglandin synthetase is a major source of alkyl hydroperoxides produced during normal animal metabolism. Therefore, tissues which possess high levels of prostaglandin synthetase should be particularly active in peroxidatic BP oxidation.

The peroxidatic oxidation of BP by both the Tween 20 solubilized preparation and metHb generates a derivative which is capable of covalent attachment to exogenous tRNA. The Tween 20 solubilized preparation was used to facilitate phenol extraction and phase separation during the workup. Sivarajah et al. have recently demonstrated that prostaglandin synthetase dependent oxidation of radiolabeled BP causes the covalent attachment of radioactivity to exogenous DNA (Sivarajah et al., 1978). These authors have used prostaglandin synthetase preparations from sheep seminal vesicles, guinea pig lung, and human platelets with similar results. The electrophilic derivative of BP appears to be short-lived. In the present case, maximal binding occurs when the nucleic acid is present with the enzyme and BP prior to the addition of 20:4. If the nucleic acid is added 15 min after the 20:4, very little covalent binding of BP to tRNA occurs. This also indicates that the quinone metabolites are not responsible for the binding to tRNA. These compounds are stable so the same level of binding should be obtained regardless of when the nucleic acid is added.

Although the incubations carried out in the presence of tRNA indicate that prostaglandin synthetase metabolically activates BP to electrophilic derivatives, our recent attempts to demonstrate the production of mutagens from BP under similar conditions have been unsuccessful (Marnett et al., 1978). BP and prostaglandin synthetase were incubated with *Salmonella typhimurium* strain TA 98. TA 98 was chosen because it has been reported to detect 6-hydroxy-BP as a mutagen. Control experiments indicated that the bacterial suspension inhibited neither prostaglandin biosynthesis nor BP oxidation. However, incubations designed to give maximum BP oxidation and performed in the presence or absence of metHb resulted in no increase in the reversion frequency of the bacteria to histidine independence. Similar negative results were obtained with tester strain TA 100. It is possible that covalent binding of the electrophilic derivatives of BP to bacterial DNA occurs but does not constitute a mutagenic event. Alternatively, the electrophilic derivatives of BP, which are produced outside of the bacteria, may be too unstable to diffuse inside the bacteria and bind to DNA.

[3] Additional hydroperoxides undoubtedly occur in animal tissue as a result of lipid peroxidation and radiation-induced peroxidation. We have limited the present discussion to hydroperoxides which appear to be formed by defined and regulable pathways of animal metabolism. This does not imply that, for instance, lipid hydroperoxides are not important substrates for the heme protein peroxidase activity. In fact, Aust and co-workers have demonstrated co-oxidations similar to the ones we have observed using phospholipid hydroperoxides and a variety of heme proteins (S. D. Aust, personal communication).

Acknowledgments

We are grateful to John T. Johnson for the LC profile in Figure 1 and to William R. Pagels for the iodometric analysis of the hydroperoxides. The tRNA binding studies were skillfully performed by Melissa Tuttle. We acknowledge helpful discussions with Dr. Charles M. King regarding the latter experiments.

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