Effect of Inducers and Inhibitors of Monooxygenase on the Hydroxylation of Prostaglandins in the Guinea Pig

EVIDENCE FOR SEVERAL MONOOXYGENASES CATALYZING $\omega$ AND $\omega$-1-HYDROXYLATION*

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The incubation of prostaglandins (PG's) with liver microsomes from guinea pigs treated with inducers of monooxygenase (1,1,1-trichloro-2,2-bis(p-chlorophenyl)ethane (DDT), benzo[a]pyrene (benzpyrene), or a mixture of chlorinated biphenyls (Aroclor 1254)) exhibited marked elevation of 19-hydroxylation of PGE$_2$, PGE$_3$, PGA$_1$, and PGA$_2$, without affecting significantly 20-hydroxylation. However, with respect to effects on hydroxylation of a variety of xenobiotics, benzpyrene and Aroclor treatments differed markedly; whereas Aroclor treatment elevated the demethylation of ethylmorphine, benzphetamine, and $p$-chloro-$N$-methylamline (PCMA), benzpyrene treatment had no effect on demethylation of ethylmorphine and only a marginal effect on that of PCMA. Both inducers elevated benzpyrene hydroxylation. By contrast, treatment with phenobarbital did not affect the hepatic microsomal PG's hydroxylation, although the hydroxylation of benzpyrene and the demethylation of ethylmorphine, benzphetamine, and PCMA were enhanced. Also, the hydroxylation of PG's by kidney cortex microsomes was not affected by either benzpyrene or Aroclor treatment.

Inhibitors of monooxygenase were used to help delineate the type of monooxygenases induced. At low levels of $\alpha$-naphthoflavone (ANF), benzpyrene hydroxylation in control- and Aroclor-treated guinea pigs was only little affected; by contrast, the same concentration of ANF markedly inhibited benzpyrene hydroxylation in benzpyrene-treated guinea pigs. On the other hand, metyrapone was most inhibitory in control guinea pigs. Support for the conclusion that benzpyrene induces in the guinea pig a hepatic monooxygenase with different characteristics than that found in control animals was provided by the observation that ANF (10 $\mu$M) inhibited PGE$_2$ hydroxylation more pronouncedly in liver microsomes from benzpyrene-treated than from Aroclor-treated guinea pigs or controls. In addition, in benzpyrene and Aroclor-treated guinea pigs, ANF inhibited the $\omega$-1-hydroxylation more pronouncedly than that of $\omega$-hydroxylation. By contrast, metyrapone appeared to inhibit $\omega$-hydroxylation more effectively than ($\omega$-1)-hydroxylation.

These results indicate that in the guinea pig, hydroxylation of PG's at the $\omega$ (20-) and $\omega$-1 (19-) positions is catalyzed by different monooxygenases and that the inducers tested affect several hepatic monooxygenases with different specificities toward xenobiotics; however, with respect to PG's only the enzyme(s) involved in the 19-hydroxylation is affected.

Previous studies showed that endogenous and exogenous prostaglandins (PG's$^1$) in animals and man are excreted in the urine as dicarboxylic acids (1-4). These observations led to the speculation that the first step in the formation of the $\omega$-carboxy derivatives of PG's is hydroxylation at the $\omega$ carbon (1). In turn, it was reasonable to assume that the microsomal cytochrome P-450 monooxygenase was the enzyme system catalyzing this hydroxylation. In fact, subsequent studies (5-10) demonstrated that microsomal monooxygenases of liver, kidney cortex, and adrenals catalyze the hydroxylation (at C$_9$ and C$_{10}$) of prostaglandins (Fig. 1). Recently, it was also shown that rabbit lung and liver microsomes $\omega$-hydroxylate PG's and that pregnancy and progesterone treatment dramatically elevate the 20-, but not the 19-, hydroxylation (11, 12).

Treatment of animals with a variety of structurally unrelated compounds (referred to as inducers) increases the microsomal monooxygenase activity toward numerous substrates (13). In addition, it was shown that certain inducers of monooxygenase enhance preferentially the hydroxylation of some substrates and not of others (14-18). Remarkably, some inducers enhance the hydroxylation at specific positions on the same molecule, whereas other inducers enhance hydroxylation at other positions (19-22). These and other findings led to the conclusion that the different sites of hydroxylation are catalyzed by different species of cytochrome P-450's (23, 24). Evidence involving the isolation of different P-450 fractions and reconstitution of the monooxygenase systems substantiated that the specificity toward given substrates or given site on a substrate resides in the P-450 molecule, whereas little or no specificity appears to be inherent in other components of the monooxygenase system (25-32).

The site of hydroxylation of PG's (at C$_9$ or C$_{10}$) would tend to direct the subsequent course of metabolism and disposition.

$^*$ The abbreviations used are: PG's, prostaglandins; PGA$_1$, and PGA$_2$, prostaglandins A, and B$_2$; PGE$_1$, and PGE$_2$, prostaglandins E, and E$_2$; PGB$_1$, and PGB$_2$, prostaglandins B, and B$_2$; 19-OH-PGB's, 19-hydroxy-PGB's; 20-OH-PGB's, 20-hydroxy-PGB's; DDT, 1,1,1-trichloro-2,2-bis(p-chlorophenyl)ethane; benzpyrene, benzo[a]pyrene; Aroclor 1254, a mixture of chlorinated biphenyls; ANF, $\alpha$-naphthoflavone; HPLC, high pressure liquid chromatography; GC, thin layer chromatography; GC, gas chromatography; MS, mass spectrometry; AIFS, absorbance units full scale; PCMA, $p$-chloro-$N$-methylamline.

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The present investigation examines the effects of several inducers of monooxygenase on the rate and mode of hydroxylation of PGE's and PGF's by hepatic and renal cortex microsomes in the guinea pig. Also, the effect of these inducers on the hydroxylation of certain classical xenobiotic substrates is examined in order to determine whether these inducers affect the substrate specificity of the monooxygenase. These latter experiments were of interest since information was sought to determine whether inducers enhance the same monooxygenases which oxidize both xenobiotics and PGE's. Furthermore, inhibitors of monooxygenases were employed to help establish whether the ω- and (ω-1)-hydroxylations of PGE's are catalyzed by the same or by different monooxygenases.

**Materials and Methods**

[(5,6,8,11,12,14,15,16)-3H]Prostaglandin E1, 89.5 Ci/mmol, was purchased from New England Nuclear (Boston, Mass.). [(5,6,8,11,12,14,15)-3H]Prostaglandin E2, 130 to 160 Ci/mmol, was purchased from New England Nuclear (Boston, Mass.) and from Amersham/Searle (Chicago, Ill.), respectively; [(5,6,8,11,12,14,15,16-3H]Prostaglandin A2, 160 Ci/mmol, was purchased from New England Nuclear (Boston, Mass.) and from Amersham (Chicago, Ill.). Prostaglandin A2, 20-OH-PGE2, methyl esters (Upjohn Co.), this was achieved by adding 0.1 ml of 10 n KOH to a solution of the respective methyl ester in 6 ml of aqueous methanol (1:1) and allowing the mixture to stand at room temperature for 19 h. The solution was acidified with 1 n HCl to pH 2, evaporated under a stream of N2 to 2 ml, and extracted with ethyl acetate. The ethyl acetate phase was evaporated to dryness under a stream of N2. The resulting residue was in the form of 19-OH-PGE2; or 19-OH-PGB2, respectively; structures were established by HPLC and, following esterification with diazomethane and silylation with t-butyldimethylsilyl chloride and trimethylsilyl chloride, by GC/MS. 20-OH-PGE2, the silylated derivatives of 20-OH-PGB2, were prepared as with the 19-OH-PGB2's and a mass fragmentation pattern obtained. To obtain additional quantities of 19-OH-PGB2 and 19-OH-PGB2, these compounds were extracted from human semen and purified by HPLC (39). Their identity was established by comparing their chromatographic characteristics as free acids and methyl esters in HPLC with the above described authentic 19-OH-PGB2's and by mass fragmentation patterns and GC retention times of their silylated methyl ester derivatives. Once identified, the isolated 19-OH-PGB2's were used as authentic compounds to help characterize hydroxylated PG's from incubation studies.

**Animals**

Male Hartley strain albino guinea pigs weighing 350 to 400 g were obtained from Elm Hill Farm (Chelmsford, Mass.). Treatment is described in the tables.

**Microsomes**

Liver or kidney microsomes were prepared as previously described (9, 40) and were used fresh or stored under a layer of 1.15% KCl at −70°C. Under these conditions of storage, activity remained unchanged for several weeks. Protein determinations were carried out by a modified (41) Lowry procedure (42), using bovine serum albumin as a standard.

**Incubation**

(a) Ethylmorphine (16 μmol), benzetamine (2 μmol), or p-chloro-N-methylaniline (3 μmol) was incubated in 2 ml final volume in glass scintillation vials containing Tris (100 μmol), pH 7.5 buffer, MgCl2 (10 μmol), semicarbazide (1 μmol), NADPH-generating system (NADP, 1 μmol; glucose 6-phosphate, 9 μmol; and glucose-6-phosphate dehydrogenase, 2 units), and microsomal suspension, 0.1 ml in aqueous 1.15% KCl (protein concentration in Table I). The reaction was started by adding the NADPH-generating system in buffer and incubating in a Dubnoff incubator with shaking for 20 min at 37°C. Reaction was stopped by adding 0.6 ml of 20% ZnSO4 followed by 0.6 ml of aqueous 1.15% KOH to a solution of the respective methyl ester in 6 ml of aqueous methanol (1:1) and allowing the mixture to stand at room temperature for 19 h. The solution was acidified with 1 n HCl to pH 2, evaporated under a stream of N2 to 2 ml, and extracted with ethyl acetate. The ethyl acetate phase was evaporated to dryness under a stream of N2. The resulting residue was in the form of 19-OH-PGE2; or 19-OH-PGB2, respectively; structures were established by HPLC and, following esterification with diazomethane and silylation with t-butyldimethylsilyl chloride and trimethylsilyl chloride, by GC/MS. 20-OH-PGE2, the silylated derivatives of 20-OH-PGB2, were prepared as with the 19-OH-PGB2's and a mass fragmentation pattern obtained. To obtain additional quantities of 19-OH-PGB2 and 19-OH-PGB2, these compounds were extracted from human semen and purified by HPLC (39). Their identity was established by comparing their chromatographic characteristics as free acids and methyl esters in HPLC with the above described authentic 19-OH-PGB2's and by mass fragmentation patterns and GC retention times of their silylated methyl ester derivatives. Once identified, the isolated 19-OH-PGB2's were used as authentic compounds to help characterize hydroxylated PG's from incubation studies.

(b) [(5,6,11,12,14,15)-3H]Prostaglandin A2, 160 Ci/mmol, was purchased from New England Nuclear (Boston, Mass.) and from Amersham/Searle (Chicago, Ill.), respectively; [(5,6,8,11,12,14,15-3H]Prostaglandin E1, 89.5 Ci/mmol, was purchased from New England Nuclear (Boston, Mass.) and from Amersham/Searle (Chicago, Ill.), respectively; [(5,6,8,11,12,14,15,16-3H]Prostaglandin E2, 130 to 160 Ci/mmol, was purchased from New England Nuclear (Boston, Mass.) and from Amersham (Chicago, Ill.). Prostaglandin A2, 20-OH-PGE2, methyl esters (Upjohn Co.), this was achieved by adding 0.1 ml of 10 n KOH to a solution of the respective methyl ester in 6 ml of aqueous methanol (1:1) and allowing the mixture to stand at room temperature for 19 h. The solution was acidified with 1 n HCl to pH 2, evaporated under a stream of N2 to 2 ml, and extracted with ethyl acetate. The ethyl acetate phase was evaporated to dryness under a stream of N2. The resulting residue was in the form of 19-OH-PGE2; or 19-OH-PGB2, respectively; structures were established by HPLC and, following esterification with diazomethane and silylation with t-butyldimethylsilyl chloride and trimethylsilyl chloride, by GC/MS. 20-OH-PGE2, the silylated derivatives of 20-OH-PGB2, were prepared as with the 19-OH-PGB2's and a mass fragmentation pattern obtained. To obtain additional quantities of 19-OH-PGB2 and 19-OH-PGB2, these compounds were extracted from human semen and purified by HPLC (39). Their identity was established by comparing their chromatographic characteristics as free acids and methyl esters in HPLC with the above described authentic 19-OH-PGB2's and by mass fragmentation patterns and GC retention times of their silylated methyl ester derivatives. Once identified, the isolated 19-OH-PGB2's were used as authentic compounds to help characterize hydroxylated PG's from incubation studies.

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**Effect of treatment of guinea pigs with inducers of monooxygenase on hepatic microsomal monooxygenase activity**

Values represent an average of duplicate incubations ± S.E. of incubations from several animals (n). Demethylations: nanomoles of HCHO/mg of protein; benzpyrene hydroxylation: picomoles/min/mg of protein; P-450: nanomoles/mg of protein. Demethylations: incubations with ethylmorphine, benzphetamine, and p-chloro-N-methylaniline. Each incubation contained 0.9 to 1.3 mg of microsomal protein and an NADPH-generating system. Hydroxylation: incubations with [1^4C]PGA (Exp. 2 = 37.0 nmol; 332,000 dpm; Exp. 3 = 40.8 nmol; 423,180 dpm), 0.2 to 0.35 mg of protein and NADPH (0.5 mm/mg of protein; P-450: nanomoles/mg of protein. Demethylations: allyl in water (75 mg/kg/day in two divided doses) for 4 days; animals incubations with ethylmorphine, benzphetamine, and p-chloro-N-methylaniline. Each incubation contained 0.9 to 1.3 mg of microsomal protein and an NADPH-generating system. Hydroxylation: incubations with [1^4C]PGA (Exp. 2 = 37.0 nmol; 332,000 dpm; Exp. 3 = 40.8 nmol; 423,180 dpm), 0.2 to 0.35 mg of protein and NADPH (0.5 mm/mg of protein; P-450: nanomoles/mg of protein. Demethylations: allyl in water (75 mg/kg/day in two divided doses) for 4 days; animals killed 16 h after last injection. Benzpyrene and Aroclor 1254: injected intraperitoneally in 0.4 ml of corn oil once at 40 mg/kg and 500 mg/kg, respectively; animals killed 48 h late. Controls: received the vehicles only. T/C = ratio of value from treated to controls.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Ethylmorphine</th>
<th>Benzphetamine</th>
<th>p-Chloro-N-methylaniline</th>
<th>Benzphetamine</th>
<th>P-450*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Exp. 1</td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Control</td>
<td>3.8 ± 0.5</td>
<td>4.6 ± 0.6</td>
<td></td>
<td>0.65 ± 0.02</td>
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<tr>
<td>DDT (4)</td>
<td>7.1 ± 1.0</td>
<td>1.9</td>
<td>11.7 ± 1.3</td>
<td>0.94 ± 0.05</td>
<td>1.4</td>
</tr>
<tr>
<td>Exp. 2</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>3.1 ± 0.2</td>
<td>3.9 ± 0.2</td>
<td></td>
<td>6.3 ± 0.2</td>
<td></td>
</tr>
<tr>
<td>Phenobarbital (5)</td>
<td>16.0 ± 1.2</td>
<td>5.1</td>
<td>210.0 ± 9.4</td>
<td>12.6 ± 1.1</td>
<td></td>
</tr>
<tr>
<td>Exp. 3</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>1.9 ± 0.1</td>
<td>3.0 ± 0.1</td>
<td></td>
<td>4.0 ± 0.2</td>
<td></td>
</tr>
<tr>
<td>Phenobarbital (5)</td>
<td>1.9 ± 0.2</td>
<td>1.0</td>
<td>4.5 ± 0.2</td>
<td>4.8 ± 0.2</td>
<td></td>
</tr>
<tr>
<td>Aroclor (5)</td>
<td>3.7 ± 0.4</td>
<td>1.9</td>
<td>8.5 ± 0.7</td>
<td>7.8 ± 0.8</td>
<td></td>
</tr>
</tbody>
</table>

* Peak of absorption of the reduced P-450-CO complex. Exp. 1: control, 451 nm; DDT, 451 nm. Exp. 2: control, 451 nm; phenobarbital, 451 nm. Exp. 3: control, 450 nm, benzpyrene, 450 nm, Aroclor, 450 nm.

**Gas Chromatography-Mass Spectrometry (GC/MS)**

This was carried out using a Perkin-Elmer gas chromatograph model 990 with a flame ionization detector coupled to a Hitachi Perkin-Elmer mass spectrometer model RMU-GL. The GC was equipped with a silanized glass column (6 feet × 0.25 inch) packed with 1% SE30 on Gas-chrom Q (100 to 120) and was operated under the following conditions: column temperature was kept at 220°C for 10 min after injection, followed by a 16°C/min program to 300°C or run isothermally at 250°C; injection port, 250°C; interface, 270°C; carrier gas, helium (30 ml/min). The mass spectrometer was operated under the following conditions: ionizing voltage, 70 eV; accelerating voltage, 2.2 kV; source, 210°C.

**High Pressure Liquid Chromatography (HPLC)**

The high pressure liquid chromatograph obtained from Waters Associates (ALC-GPC 204) was equipped with dual wavelength UV detector (model 440), solvent delivery system (model 600A), injector (model U6K), and solvent flow programmer (model 660). Reverse phase chromatography was carried out with a "fatty acid" column (4 mm × 30 cm) (Waters Associates) using acetonitrile and 1% aqueous acetic acid for the free acids or acetonitrile and water for the methyl esters.

**Detection, Isolation, and Quantification of Metabolites**

Detection—Usually the evaporated ethyl acetate extract containing PGB's and metabolites was dissolved in 2 to 3 ml of 0.1 N NaOH. The basic solution was extracted with 3 × 3 ml of chloroform and the organic phase was discarded (there was little or no radioactivity in the organic phase). The aqueous phase was then acidified with 1 N HCl to pH 2 to 3 and extracted with 3 × 4 ml of ethyl acetate. The ethyl acetate phase was evaporated to dryness under a stream of N₂ at room temperature. The residue was dissolved in methanol and the absorbance at 278 nm was measured to assess total amount of PGB derivatives (ε = 27.2 mM⁻¹ cm⁻¹). The methanol was evaporated to dryness under N₂ at room temperature and the residue was dissolved in 40 to 100 µl of methanol. The amount of polar metabolite(s) was determined by tlc (see "Quantitation"). This information was utilized to choose an appropriate aliquot for HPLC (see figures). Since PGB derivatives have a λₘₐₓ = 278 nm, monitoring in HPLC was carried out at 280 and 254 nm and occasionally at 313 nm and peaks having a higher extinction at 280 nm than at 254 or 313 nm were assumed to represent PGB's and PGB derivatives. Particular attention was paid to peaks with retention times corresponding to 19-OH-PGB's and 20-OH-PGB's. In early experiments, to establish that the UV-absorbing compounds were tritium-labeled PG metabolites, equal (5.0 ml) fractions were collected from the HPLC. The fractions were dried under...
a stream of nitrogen and each dissolved in 1 ml of methanol. To each 50-µl aliquot was added 5 ml of solution of Liquifluor (New England Nuclear) (42 ml/liter of toluene) and the radioactivity was monitored in a Packard Tri-Carb scintillation spectrometer.

Isolation of Metabolites—To obtain sufficient quantities of metabolites for identification purposes, extracts from similar incubations were first combined and processed by HPLC as described above. The eluted fractions were evaporated to dryness under nitrogen flow at room temperature and dissolved in methanol. The UV absorption spectrum was obtained in a Gilford spectrophotometer. To examine the purity of a given fraction, aliquots were again analyzed by HPLC.

Identification of Metabolites—Aliquots of solutions of PG metabolites, which were isolated as above, were co-injected with a sample of an authentic 19-OH-PGB. The appearance of a single peak representing additive peak intensities was assumed to indicate identical retention times of the metabolite and the corresponding authentic compound. Subsequently, the isolated metabolite(s) were esterified with diazomethane (generated from N-methyl-N-nitrosourea) and their retention time in HPLC was compared with that of esterified 19-OH-PGB and 20-OH-PGB. Portions of the esterified PG's (metabolites or authentic compounds) were silylated with trimethylsilyl chloride in dry pyridine or with t-butyldimethylsilyl chloride in the presence of imidazole for 48 to 72 h at room temperature. To extract the t-butyldimethylsilyl derivatized PG's, water was added followed by three extractions with hexane. The hexane extract was evaporated to dryness under N₂ gas, the residue was dissolved in methanol, and recoveries were calculated using ε at 278 nm = 27.2 mM⁻¹·cm⁻¹ (9, 45). Aliquots of these solutions which were subjected to HPLC revealed a dramatic prolongation of retention time of the silylated prostaglandin derivatives over that of the corresponding methyl esters. This indicated conversion to highly nonpolar substances as would be expected from the corresponding silyl ethers.

Quantitation—Residual extracts, which did not undergo HPLC (for work-up see "Incubation"), were dissolved in a few drops of methanol and were chromatographed on thin layer plates (previously activated by heating at 110°C for 30 min). The solvent system consisted of ethyl acetate/acetic acid/2,2,4-trimethylpentane/water (110:20:20:100). The plates were dried in air and were scanned on a Vangard model 930 thin layer scanner at high sensitivity (100 D) at a rate of 8 inches/h. Usually, the zones containing the radioactivity were collected by scraping the gel with the help of a razor blade into scintillation vials. One milliliter of methanol was added to the vials which were swirled, and 5 ml of Liquifluor (New England Nuclear) was added. The radioactivity was determined in a Packard Tri-Carb scintillation spectrometer. To examine the purity of a given fraction, aliquots were again analyzed by HPLC.

RESULTS AND DISCUSSION

Identification of Products

PGE₁ and PGA₁ Metabolites

In previous studies we determined that incubation of PGE₁ and PGA₁ with guinea pig liver and kidney cortex microsomes yielded primarily 19-hydroxy derivatives which, following base treatment, were characterized as 19-hydroxy-PGB₁ by HPLC and GC/MS (8, 9). Hence, in the present investigation the nature of the metabolites of PGE₁ and PGA₁, as being the 19-hydroxylated products was merely confirmed with HPLC by co-chromatography of the free acid and of the methyl ester of the metabolites with similarly derivatized authentic compounds.

The major polar PGE₁ and PGA₁ metabolites (after base treatment) from incubations with liver microsomes of control, benzpyrene-treated, and Aroclor-treated guinea pigs had identical retention in HPLC as 19-OH-PGB₁. Also the derived methyl ester of the isolated metabolites from these incubations had the same retention time in HPLC as that of the methyl ester of authentic 19-OH-PGB₁. A typical HPLC scan of the methyl ester of the combined eluted fractions from the major polar metabolite from incubations of PGE₁ and PGA₁ with liver microsomes of control and Aroclor 1254-treated guinea pigs is shown in Fig. 2; as can be seen the metabolite ester had identical retention to the methyl ester of 19-OH-PGB₁. A minor, less polar peak (longer retention time than 19-OH-PGB₁) was always observed. This metabolite was assumed to result from 20-hydroxylation of PGA₁ and PGE₁, since after base treatment its chromatographic characteristics were identical with that of 20-OH-PGB₁; also after esterification with CH₂N₂O this metabolite co-chromatographed with the methyl ester of authentic 20-OH-PGB₁.

PGE₂ and PGA₂ Metabolites

The metabolites of PGE₂ and PGA₂ from incubations with hepatic microsomes have not been previously characterized. Hence, we carried out a more rigorous identification of the

![Fig. 2. HPLC of the methyl ester of the isolated base-treated metabolite from PGE₁ and PGA₁. Incubations with liver microsomes of control and Aroclor 1254-treated guinea pigs as compared to methyl ester of 19-OH-PGB₁. The combined fractions of the isolated major polar metabolite from PGE₁ and PGA₁ incubations were esterified with diazomethane, dissolved in methanol, and subjected to HPLC. Conditions of HPLC: 25% acetonitrile: 75% H₂O (eluting solvent); flow rate, 2 ml/min; chart speed, 1 cm/min; p.s.i. = 1500. AUFS = 0.01. The metabolite of interest with a higher absorbance at 280 nm (lower scan) than at 254 nm (upper scan) is indicated with an arrow. The two scans are offset by 1.8 cm. A, 12 µl (0.99 µg) of methyl ester of metabolite; B, 4 µl (0.05 µg) of the methyl ester of authentic 19-OH-PGB₁; C, co-injection of 12 µl of the methyl ester of metabolite and 4 µl (0.08 µg) of the methyl ester of 19-OH-PGB₁.](http://www.jbc.org/)
major metabolites by HPLC, GC, and GC/MS (see "Materials and Methods"). Identification was obtained by converting the products of incubation of PGE₂ and PGA₂ into PGB₂ derivatives.

The chromatographic mobility of the ethyl acetate extracts from incubations of PGE₂ with liver microsomes from control, benzpyrene-, and Aroclor-treated guinea pigs is shown in Fig. 3. The major polar metabolite (Peak 1) in chromatograms from the different incubations had a similar retention time to that of 19-OH-PGB₂. Hence, all of the fractions corresponding to Peak 1 were collected, combined, and analyzed by HPLC (Fig. 4); as can be seen, the polar metabolite had identical retention time to that of authentic 19-OH-PGB₂. Similar results were obtained with the combined fractions of the polar metabolite of PGA₂ isolated from the various incubations (Fig. 5); again the major polar metabolite had identical retention time to 19-OH-PGB₂, established by co-injection with the authentic derivative.

To characterize further the polar metabolite, the combined fractions containing the polar metabolite from incubations of PGE₂ and PGA₂ were esterified and analyzed by HPLC (Fig. 6). The methyl ester of this metabolite had identical retention time to that of the methyl ester of 19-OH-PGB₂. Furthermore, the t-butyldimethylsilyl ether methyl ester of the metabolite, which had a markedly longer retention time in HPLC (requiring 90% acetonitrile for elution) than the methyl ester, had similar retention to that of similarly derivatized 19-OH-PGB₂. Also, both silylated derivatives had identical retention times on GC, exhibiting on co-injection a single peak. Last, the mass fragmentation pattern of the derivatized metabolite was similar to that of a derivatized authentic 19-OH-PGB₂ (Fig. 7, A and B). The spectra exhibited a molecular ion at m/e 592 and a peak at m/e 535 (M⁻[t-buty]). Other fragments were at m/e 503 (M⁺[57 + methanol]); 490 (M⁺[t-butyldimethylsilyl alcohol, 132]; 429 (M⁺[132 + CH₂OH]); 297 (M⁺[2 × 132 + 31]). The base peak was at m/e 71. As expected from PGB₂ derivatives, the above peaks were 2 mass units lower than those observed with similarly derivatized 19-OH-PGB₁ derivatives (9). It is of interest that by contrast to mass spectrum of PG₁ trimethylsilyl ether methyl ester, the trimethylsilyl ether methyl ester of authentic 19-OH-PGB₂ did not exhibit the expected (47) fragment at m/e = 390 [M⁺-(CH₂CH₂OTMSi + 1)], although a fragment at m/e = 117 (CH₂CH₂OTMSi) was evident (spectra not shown).

These results demonstrate that incubation of various PG's with liver microsomes from control guinea pigs or from guinea pigs treated with a variety of inducers yielded primarily the corresponding 19-hydroxy-PG derivatives.

Effects of Inducers

The effect of inducers of monoxygenase on the metabolism of certain classical xenobiotic substrates of hepatic microsomal monoxygenase was compared with effects on PG hydroxylation in order to gain information on: (a) whether these inducers affect PG's metabolism and whether the effects on rate of metabolism of PG's parallel effects on metabolism of xenobiotic substrates and (b) whether the inducers increase PG's hydroxylation at both 19- and 20-positions or whether the hydroxylation is preferentially enhanced at one site of the prostaglandin molecule.

*p,p'-DDT*

Results show that the administration of *p,p'-DDT* elevated the hepatic microsomal hydroxylation of PGA₂ to a similar extent to that of demethylation of benzphetamine, whereas a slightly lower increase in demethylation of ethylmorphine was observed (Table I). The increase in metabolism also paralleled an increase in levels of constituents of the monoxygenase, NADPH-cytochrome c reductase (not shown), and cytochrome P-450.
Induction of Hydroxylation of Prostaglandins by Monoxygenase

**FIG. 4.** HPLC of the major polar metabolite isolated from incubations of PGE\(_2\) with liver microsomes of control, benzpyrene-treated, and Aroclor 1254-treated guinea pigs. The chromatograms represent the combined eluted fractions containing the major polar metabolite. HPLC conditions are the same as in Fig. 3. AUFS = 0.01. A, 5 \(\mu\)l of methanolic solution of the isolated major polar metabolite (0.08 \(\mu\)g); the arrow indicates the PGE\(_2\) metabolite of interest. Currently we have no information concerning the minor more polar peak. B, 5 \(\mu\)l of methanolic solution (0.07 \(\mu\)g) of authentic 19-OH-PGB\(_2\) in methanol obtained from semen. The small more polar (shorter retention) peak was formed from purified 19-OH-PGB\(_2\) on standing even at 0°C; we have no knowledge concerning its nature, but upon esterification with CH\(_3\)N\(_2\) this peak is chromatographically indistinguishable from the methyl ester of 19-OH-PGB\(_2\). C, co-injection of samples described in A and B (5-\(\mu\)l aliquots of each).

**FIG. 5.** HPLC of the major polar metabolite from incubations of PGA\(_2\) with liver microsomes from control, benzpyrene, and Aroclor-treated guinea pigs. The chromatogram represents the combined eluted fractions previously isolated by HPLC. The conditions are the same as in Fig. 3. AUFS = 0.01. A, 5 \(\mu\)l of methanolic solution of the isolated major polar metabolite (0.13 \(\mu\)g) containing more polar and less polar (probably 20-OH-PGB\(_2\)) contaminant. B, 5 \(\mu\)l of methanolic solution of authentic 19-OH-PGB\(_2\) (0.07 \(\mu\)g) contains a more polar contaminant. C, co-injection of 5-\(\mu\)l aliquots of samples described in A and B.

**FIG. 6.** HPLC of the methyl ester of the major polar metabolite isolated from incubations of PGE\(_2\) and PGA\(_2\) with liver microsomes from control, benzpyrene-treated, and Aroclor 1254-treated guinea pigs. The combined fractions of the isolated major polar metabolite (see Figs. 4 and 5) were esterified with diazomethane, dissolved in methanol, and subjected to HPLC analysis. conditions are as described in Fig. 2. AUFS = 0.01. A, 10 \(\mu\)l (0.09 \(\mu\)g) of methyl ester of isolated metabolite; B, 8 \(\mu\)l (0.07 \(\mu\)g) of methyl ester of authentic 19-OH-PGB\(_2\); C, co-injection of 10 \(\mu\)l of A and 8 \(\mu\)l of B.
Benzpyrene and Aroclor 1254

Effects on Hepatic Metabolism of Xenobiotics—The administration of benzpyrene increased the hepatic microsomal oxidative metabolism of benzphetamine and benzpyrene but not that of ethylmorphine and only marginally increased p-chloro-N-methylaniline demethylation (Table I). By contrast Aroclor 1254 increased the metabolism of the four substrates tested and the increases with Aroclor were higher than those observed after benzpyrene treatment (Table I).

Effects on Hepatic Metabolism of PG’s—Contrary to the relative small enhancement of benzpyrene treatment on the metabolism of xenobiotics, a pronounced increase on hepatic metabolism of the four PG’s tested (PGE1, PGE2, PGA1, and PGA2) was observed (Table II). Similarly, treatment with Aroclor 1254 increased the rate of PG’s hydroxylation; however, whereas Aroclor produced a more pronounced effect than benzpyrene on xenobiotic metabolism, benzpyrene was about equal or more effective than Aroclor on PG’s hydroxylation (Table II).

Effects on Renal Cortex Metabolism of PG’s—Neither benzpyrene nor Aroclor treatment affected PGE1 hydroxylation by renal cortex microsomes, yielding similar amounts of hydroxylated products (nanomoles/mg of microsomal protein/90 min), in controls 11.8 ± 0.9, in benzpyrene treated 11.4 ± 0.2, and in Aroclor-treated 12.3 ± 1.0.

Phenobarbital

Treatment with phenobarbital exhibited a substantially more pronounced induction of demethylation of ethylmorphine and benzphetamine than treatment with DDT, benzpy-

![Fig. 7. Mass fragmentation patterns of t-butyldimethylsilyl ether methyl ester of pooled, HPLC-purified (see Fig. 6A) metabolite from PGE2 and PGA2 incubations (upper frame) and of similarly derivatized 19-OH-PGB2 isolated from human semen (lower frame). The GC/MS described under "Materials and Methods" demonstrated identical GC retention of the two compounds.](http://www.jbc.org/)

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**Table II**

**Effect of treatment of guinea pigs with DDT, benzpyrene, and Aroclor 1254 on prostaglandin hydroxylations by hepatic microsomes**

Values represent average ± S.E. of nanomoles of hydroxylated product/30 min/mg of protein from incubations of microsomes from several animals (number of animals in parentheses), each incubation run in duplicate. T/C = ratio of value from treated to controls. Treatment as in Table I. Each 1-ml incubation contained 2.1 to 4.7 mg of microsomal protein and [3H]PG (1 mM, 0.1 to 1 μCi) except with PGA, where 1.5 mM was used. Incubations were carried out for 30 min (linearity up to 45 min was previously established).

<table>
<thead>
<tr>
<th>Treatment</th>
<th>PGE1</th>
<th>T/C</th>
<th>PGA1</th>
<th>T/C</th>
<th>PGE2</th>
<th>T/C</th>
<th>PGA2</th>
<th>T/C</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control (4)</td>
<td>5.7 ± 0.4</td>
<td></td>
<td>15.3 ± 1.7</td>
<td></td>
<td>2.7</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>DDT (4)</td>
<td></td>
<td>9.8 ± 1.5</td>
<td></td>
<td>14.6 ± 1.2</td>
<td></td>
<td>1.1</td>
<td></td>
<td>19.4 ± 1.5</td>
</tr>
<tr>
<td>Phenobarbital (5)</td>
<td>10.8 ± 1.0</td>
<td></td>
<td>10.8 ± 0.7</td>
<td></td>
<td>3.5</td>
<td></td>
<td>39.5 ± 4.5</td>
<td></td>
</tr>
<tr>
<td>Control (5)</td>
<td>7.9 ± 0.3</td>
<td></td>
<td>9.7 ± 0.3</td>
<td></td>
<td>2.9 ± 0.3</td>
<td></td>
<td>4.3</td>
<td></td>
</tr>
<tr>
<td>Benzpyrene (5)</td>
<td>97.3 ± 9.3</td>
<td></td>
<td>97.3 ± 9.3</td>
<td></td>
<td>3.5</td>
<td></td>
<td>39.5 ± 4.5</td>
<td></td>
</tr>
<tr>
<td>Control (4)</td>
<td>4.7 ± 0.1</td>
<td></td>
<td>9.7 ± 0.4</td>
<td></td>
<td>3.3</td>
<td></td>
<td>6.7 ± 0.6</td>
<td></td>
</tr>
<tr>
<td>Aroclor (5)</td>
<td>11.5 ± 1.3</td>
<td></td>
<td>11.5 ± 1.3</td>
<td></td>
<td>2.4</td>
<td></td>
<td>17.0 ± 0.9</td>
<td></td>
</tr>
</tbody>
</table>

* "p ≤ 0.005.
* Animals from a different experiment than the one used in PGE1 metabolism; only four treated versus four controls.
* "p ≤ 0.001.
* Same controls were used for benzpyrene and Aroclor-treated guinea pigs.
Induction of Hydroxylation of Prostaglandins by Monoxygenase

Effects of Inducers on Site (19- or 20-) of Hydroxylation of PG's

The question whether the inducers affect both 19- and 20-hydroxylation of PG's was examined. Using reversed phase HPLC we observed that the various inducers, p,p'-DDT, benzpyrene, and Aroclor 1254, enhanced almost exclusively the major pathway, 19-hydroxylation (Figs. 1 to 4); thus, induction increased both the total extent of hydroxylation and the ratio of 19- to 20-hydroxylation.

Effects of Inhibitors of Monoxygenase α-Naphthoflavone (ANF) and Metyrapone on the Hepatic Microsomal Hydroxylation of Benzpyrene and PGE

Inhibitors of monoxygenase often have been used to attempt to distinguish the characteristics of the P-450 involved, i.e. is the reaction catalyzed by a "constitutive" P-450 or by P-448. Thus, previous studies showed that ANF stimulated or did not inhibit benzpyrene oxidation in control and phenobarbital-treated rat liver microsomes but markedly inhibited this hydroxylation by liver microsomes from 3-methylcholanthrene-treated rats (48-50). On the other hand, metyrapone appears to inhibit hepatic monoxygenase activities in control and phenobarbital-treated animals (50-52). These findings indicated that inhibitors could be used to distinguish cytochrome P-450 (phenobarbital-treated) from cytochrome P-448 (P-450) (3-methylcholanthrene-treated) mediated reactions. Hence, in the current study these inhibitors of monoxygenase were used in an attempt to obtain information on the nature of induction of guinea pig hepatic monoxygenase (type of cytochrome P-450 formed) by benzpyrene and Aroclor 1254.

Results show that low ANF concentration (1 μm) had little or no effect on benzpyrene hydroxylation in Aroclor-treated or control guinea pigs but produced a marked inhibition of benzpyrene hydroxylation in benzpyrene-treated guinea pigs (Table III). Higher levels of ANF (50 μm) inhibited benzpyrene hydroxylation in controls and treated animals. On the other hand, metyrapone was much more inhibitory in control guinea pigs than in benzpyrene- or Aroclor-treated guinea pigs. These results and those of Table I and II demonstrate that induction in the guinea pig appears to mimic that in the rat; namely, benzpyrene treatment (probably like 3-methylcholanthrene or benzpyrene in the rat) produces a different monoxygenase than the one present in controls, whereas Aroclor 1254 enhances both monoxygenases (53, 54).

Further support for the above conclusion is evident from studies on the effect of ANF and metyrapone on PGE1 hydroxylation by liver microsomes from control, benzpyrene-treated, and Aroclor-treated guinea pigs (Table IV). PGE1 hydroxylation sensitivity to ANF in the order of increasing sensitivity was: controls (least), Aroclor, and benzpyrene (most). Thus, 10 μM ANF had low effect (21% inhibition) in controls, more inhibition (37%) in Aroclor-treated, and pronounced inhibition (63%) in benzpyrene-treated guinea pigs. It appears that ANF inhibits specifically only a portion of the P-450's-catalyzed reaction, since increasing the concentration of ANF 5-fold to 50 μM increased the inhibition of PGE1 hydroxylation only slightly above that of 10 μM ANF. This might be taken as evidence that in benzpyrene-treated guinea pigs there is a higher portion of the monoxygenase which is ANF-sensitive than in Aroclor-treated guinea pigs or controls. Since metyrapone is thought to inhibit the P-450 (not the P-448), it appears to inhibit hepatic monoxygenase activities in control and phenobarbital-treated animals (50-52).

**Table III**

Effect of inhibitors of monoxygenase on benzene hydroxylation by liver microsomes from control, benzpyrene, and Aroclor 1254-treated guinea pigs

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Inhibitor</th>
<th>Phenolic products</th>
<th>% of control</th>
<th>μmol/min/mg protein</th>
<th>pmol/min/mg protein</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>Control</td>
<td>188</td>
<td>100</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>ANF (1)</td>
<td>205</td>
<td>108.3</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>ANF (50)</td>
<td>42</td>
<td>22.2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>Metyrapone (100)</td>
<td>13</td>
<td>6.8</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Benzpyrene</td>
<td>Control</td>
<td>944</td>
<td>100</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Benzpyrene</td>
<td>ANF (1)</td>
<td>191</td>
<td>78.3</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Benzpyrene</td>
<td>ANF (50)</td>
<td>36</td>
<td>14.7</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Benzpyrene</td>
<td>Metyrapone (100)</td>
<td>64</td>
<td>26.1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Aroclor</td>
<td>Control</td>
<td>335</td>
<td>100</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Aroclor</td>
<td>ANF (1)</td>
<td>399</td>
<td>92.2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Aroclor</td>
<td>ANF (50)</td>
<td>78</td>
<td>23.2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Aroclor</td>
<td>Metyrapone (100)</td>
<td>122</td>
<td>37.0</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
448)-catalyzed reactions, it is surprising that metyrapone equally inhibited PGE₁ hydroxylation by liver microsomes from control and benzpyrene-treated guinea pigs. Interestingly, metyrapone inhibited benzpyrene and PGE₁ hydroxylation to a somewhat lesser extent in Aroclor-treated than in controls or benzpyrene-treated animals. Nevertheless, although the various treatments of guinea pigs did not produce a distinct shift in λ_max of the CO-bound reduced cytochrome P-450 (Table I), benzpyrene treatment did alter the characteristics of the enzyme. Remarkably, in the guinea pig different types of xenobiotics (constitutive and induced by various inducers) catalyzed the hydroxylation at the 19-position, whereas the 20-hydroxylation was not induced and remained almost insignificant. In addition it is puzzling that although phenobarbital induced markedly the oxidation of several types of xenobiotics (ethylmorphine, benzphetamine, and benzyrene), it had little or no effect on hydroxylation of PG's. Of further interest is the observation that ANP inhibited preferentially the 19-hydroxylation of PGE₁ and had only little effect on 20-hydroxylation; conversely, metyrapone inhibited 20-hydroxylation more pronouncedly (Table IV).

**Effects of Potential Alternate Substrates of Hepatic Monoxygenase on PGE₁ Hydroxylation**

To obtain indirect evidence whether other PG's and cortisol might potentially serve as alternate substrates for the same hepatic monoxygenase, the effects of addition of PG's and cortisol, at a molar ratio of 10:1 to PGE₁, on the hydroxylation of PGE₁ were examined. Table V shows that PGA₁ and cortisol strongly inhibited PGE₁ hydroxylation. However, PGE₂ only slightly inhibited (26.7%) and PGE₃'s yielded little or no inhibition of PGE₁ hydroxylation. These results suggest that PGE₁ would be poor substrates for the monoxygenase system catalyzing PGE₁ hydroxylation. The low inhibiting effect of PGE₂ on PGE₁ metabolism was surprising since PGE₂ is metabolized effectively, although at a slower rate than PGE₁, by guinea pig liver microsomes (Table II); possibly, the relative affinity of PGE₂ for the monoxygenase is lower than that of PGE₁, PGA₁, or cortisol. Also, it is possible, although less likely, that different monoxygenases hydroxylate PGE₁, PCE₂, or PCF's. Cortisol has been previously shown to be hydroxylated at the 6β- and 2α-positions by the guinea pig liver microsomal preparations (55, 56). Whether the same enzyme system catalyzes the hydroxylation of PG's and cortisol at 2α and 6β is not known.

**TABLE V**

*In vitro effect of potential alternate substrates on PGE₁ hydroxylation by guinea pig liver microsomes*

<table>
<thead>
<tr>
<th>Additions (0.1 mM)</th>
<th>Hydroxylated product</th>
<th>% Control</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>nmol/mg protein/30 min</td>
<td></td>
</tr>
<tr>
<td>Control*</td>
<td>0.705</td>
<td>100.0</td>
</tr>
<tr>
<td>PGE₂</td>
<td>0.517</td>
<td>73.3</td>
</tr>
<tr>
<td>PAG₁</td>
<td>0.393</td>
<td>42.9</td>
</tr>
<tr>
<td>PGE₃</td>
<td>0.617</td>
<td>87.5</td>
</tr>
<tr>
<td>PGE₂0</td>
<td>0.593</td>
<td>84.1</td>
</tr>
<tr>
<td>Cortisol</td>
<td>0.276</td>
<td>39.1</td>
</tr>
</tbody>
</table>

*Control yielded 0.705 (0.716, 0.693) nmol/mg of protein/30 min, primarily 19-hydroxylated product.

**CONCLUSION**

The above results demonstrate that the guinea pig hepatic microsomal monoxygenase catalyzes effectively the 19-hydroxylation and to a minor extent the 20-hydroxylation of PG's and PAG's. The inducers tested, p,p'-DDT, benzpyrene, and Aroclor 1254, which in the rat demonstrate different types of induction, enhanced in the guinea pig only the 19-hydroxylation of PG's. By contrast, phenobarbital, which is a potent inducer of xenobiotic metabolism (Table I), exhibited little or no increase in PG's hydroxylation (Table II). Based on the degree of enhanced metabolism of different xenobiotics and PG's after induction, it appears that in the guinea pig, as in the rat and rabbit, there are several monoxygenases and that the inducers enhance the levels of these monoxygenases to a different extent. A case in point is the lack of induction by benzpyrene of ethylmorphine demethylation and the marginal effect on PCMA demethylation in the guinea pig versus the pronounced increase of PG's hydroxylation (Tables I and II). Similarly, 3-methylcholanthrene treatment in the rat failed to elevate ethylmorphine demethylation (54). Further evidence for the existence of several cytochrome P-450's has been obtained by observing on gel electrophoresis the presence of several protein bands derived from solubilized guinea pig liver microsomes (57). Also the observation that ascorbate deficiency lowers total cytochrome P-450 in the guinea pig and substantially lowers aminopyrine and p-nitroanisole demethylation and aniline hydroxylation but does not affect benzpyrene hydroxylation (58) points to the presence of several monoxygenases. We are currently investigating whether ascorbate deficiency would affect in the guinea pigs PG's hydroxylation by the hepatic monoxygenase.

Evidence that in the rat ω- and ω-1-hydroxylations of fatty acids represent activities of two enzymes has been presented (22–24, 59, 60). Thus, phenobarbital treatment enhanced only the ω-1-hydroxylation and the addition of SKF 525A, metyrapone, aminopyrine, and ANF inhibited ω-1-hydroxylation of fatty acids. It is of interest that in the guinea pig also only the (ω-1)-hydroxylation pathway of PG's was enhanced by the inducers examined and ANF inhibited preferentially this site of hydroxylation; however, by contrast to the effect of metyrapone on (ω-1) fatty acid hydroxylation in the rat, PGE₁ hydroxylation in the guinea pig was inhibited by metyrapone preferentially at the ω-position. Also of utmost interest in this regard are the observations (12) that, in the rabbit, pregnancy stimulates about 20-fold liver microsomal 20-hydroxylation of PGE₂₀, but has no effect on the 19-hydroxylation and progesterone treatment and pseudo-pregnancy have a similar, although less pronounced, effect than pregnancy on liver 20-hydroxylation. In this regard, it is also of interest that in preliminary studies we observed in rabbits that phenobarbital treatment enhances 19- and 20-hydroxylation of PG's, whereas β-naphthoflavone has little or no effect. These results strongly indicate that different monoxygenase (P-450's) are involved in the 19- and 20-hydroxylations of PG's and that exposure of animals to various conditions could specifically alter the activity of certain monoxygenases catalyzing PG's hydroxylation.

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* D. Kupper and A. D. Theoharides, unpublished observations.
Induction of Hydroxylation of Prostaglandins by Monooxygenase

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