

# Selective Activation of Some Dihydrodiols of Several Polycyclic Aromatic Hydrocarbons to Mutagenic Products by Prostaglandin Synthetase

Janet Guthrie, Iain G. C. Robertson, Errol Zeiger, Jeffrey A. Boyd, and Thomas E. Eling<sup>1</sup>

Laboratory of Molecular Genetics [J. G., I. G. C. R., E. Z.], and Laboratory of Pulmonary Function & Toxicology [J. A. B., T. E. E.], National Institute of Environmental Health Sciences, Research Triangle Park, North Carolina 27709

## ABSTRACT

The ability of prostaglandin synthetase (PGS) to cooxidize benzo(a)pyrene, benzo(a)anthracene, chrysene, and several of their dihydrodiol derivatives to mutagenic products was tested with *Salmonella typhimurium* strains TA98 and TA100. The microsomal fraction of ram seminal vesicles, a known source of PGS, in the presence of the PGS substrate arachidonic acid, metabolized benzo(a)pyrene-7,8-dihydrodiol, benzo(a)anthracene-3,4-dihydrodiol, and chrysene-1,2-dihydrodiol to mutagenic products. This activity was inhibited by the PGS inhibitor indomethacin. Unlike the PGS system, however, a cytochrome P-450-reduced nicotinamide adenine dinucleotide phosphate-dependent system, present in an Aroclor 1254-induced rat liver 9000 × g supernatant fraction, also activated the parent compounds [benzo(a)pyrene, benzo(a)anthracene, chrysene] and several other benzo(a)anthracene dihydrodiols (the 1,2-dihydrodiol, the 8,9-dihydrodiol, and the 10,11-dihydrodiol). The chrysene *trans*-3,4, *trans*-5,6, and *cis*-5,6 diols were not activated to mutagens by either system. Thus, the PGS system appears to be more selective than does the cytochrome P-450 system in the activation of polycyclic aromatic hydrocarbons to mutagenic products, activating only those dihydrodiols with adjacent double bonds in the bay region from which the bay-region diol-epoxides are formed.

## INTRODUCTION

PAH<sup>2</sup> are widespread environmental pollutants found in such sources as soot, automobile exhaust, and cigarette smoke. Metabolism of PAH is necessary for mutagenic and carcinogenic activity, and the hypothesized ultimate mutagenic and carcinogenic metabolites are the bay-region diol-epoxides (see Chart 5) formed by the oxidation of the corresponding dihydrodiols (2, 10).

An arachidonic acid-dependent PGS system is capable of cooxidizing xenobiotics (8, 18-20), including PAH (6, 8, 13-15). The PGS system is peroxidatic (6), and the cooxidation of exogenous compounds appears to occur via a free radical mechanism (4). PGS has 2 activities: (a) the cyclooxygenase

activity which catalyzes the oxygenation of arachidonic acid to the hydroperoxy endoperoxide, prostaglandin G<sub>2</sub> (9, 11, 16); and (b) the hydroperoxidase activity which catalyzes the reduction of prostaglandin G<sub>2</sub> to the hydroxy endoperoxide, prostaglandin H<sub>2</sub> (9, 11). The hydroperoxidase component is responsible for the cooxidations observed during prostaglandin biosynthesis (6, 11).

Marnett *et al.* (7) have shown that a Tween 20-solubilized enzyme preparation from RSV microsomes selectively metabolized BP-7,8-diol (but not BP, BP-4,5-diol, or BP-9,10-diol) to mutagenic derivatives using a modified Ames test when incubated with the PGS substrate arachidonic acid. These results raise 3 questions: (a) can PGS activate other PAH to mutagens; (b) will PGS activate only dihydrodiol metabolites containing adjacent double bonds in the bay region; and (c) is there a qualitative difference between the activating potential of PGS and that of the more commonly used rat liver 9000 × g supernatant (S-9)? We report experiments that are designed to answer these questions.

## MATERIALS AND METHODS

**Preparation of RSV Microsomal Fraction.** RSV were obtained from a local slaughterhouse and maintained at -80°. The tissue was chopped and then homogenized with a Polytron homogenizer (Brinkman Co., Westbury, N. Y.) (25% w/v) in cold 0.15 M KCl (adjusted to pH 7.4 with Na<sub>2</sub>HPO<sub>4</sub>). The homogenate was centrifuged at 9000 × g for 20 min at 4°, and the supernatant was recovered. Because the supernatant contained contaminating bacteria, it was necessary to sterilize this fraction by filtration. Initially, filtration was by vacuum using 0.45- and 0.2-μm pore-size Nalgene filter units (Sybron, Rochester, N. Y.). This procedure was subsequently modified to include sequential filtration through 5.0-, 1.2-, 0.8-, 0.45-, and 0.2-μm pore-size filters using a pressure filtration unit (Gelman Instrument Co., Ann Arbor, Mich.). All subsequent steps were made with sterile solutions, equipment, and techniques. The filtered supernatant was centrifuged at 100,000 × g for 30 min at 4°. The microsomal pellet was suspended in 0.15 M KCl (pH 7.4) to a protein concentration of approximately 10 mg/ml and stored at -80°.

**Preparation of Rat Liver S-9.** A 9000 × g supernatant from the livers of Aroclor 1254-induced Sprague-Dawley rats was prepared by standard procedures (1) except that a Polytron homogenizer was used, and the 0.15 M KCl was adjusted to pH 7.4 with Na<sub>2</sub>HPO<sub>4</sub> (1-g livers per 3 ml KCl). The S-9 was stored at -80° until used.

**Analytical Procedures.** PGS activity was checked prior to each experiment by the measurement of O<sub>2</sub> consumption (using a Clark oxygen electrode; Yellow Instrument Co., Yellow Springs, Ohio) after addition of arachidonic acid (400 μM) to the RSV microsomes (1 mg protein per ml) in 0.1 M phosphate buffer, pH 7.8.

Protein concentrations were determined by the method of Lowry *et al.* (3) using bovine serum albumin Fraction V as standard.

<sup>1</sup> To whom requests for reprints should be addressed.

<sup>2</sup> The abbreviations used are: PAH, polycyclic aromatic hydrocarbons; PGS, prostaglandin synthetase; RSV, ram seminal vesicles; BP-7,8-diol, benzo(a)pyrene-7,8-dihydrodiol; BP, benzo(a)pyrene; BP-4,5-diol, benzo(a)pyrene-4,5-dihydrodiol; BP-9,10-diol, benzo(a)pyrene-9,10-dihydrodiol; BA, benzo(a)anthracene; BA-1,2-diol, benzo(a)anthracene-1,2-dihydrodiol; BA-3,4-diol, benzo(a)anthracene-3,4-dihydrodiol; BA-8,9-diol, benzo(a)anthracene-8,9-dihydrodiol; BA-10,11-diol, benzo(a)anthracene-10,11-dihydrodiol.

Received July 31, 1981; accepted January 19, 1982.

**Reagents and Biochemicals.** Glucose 6-phosphate, NADP<sup>+</sup>, L-histidine-HCl, and indomethacin were obtained from Sigma Chemical Co., St. Louis, Mo. Arachidonic acid was obtained from NuChek Prep, Inc., Elysian, Minn. BP, BA, chrysene, and the diol metabolites were provided by the National Cancer Institute Chemical Repository. All PAH were dissolved in dimethyl sulfoxide (Fisher) immediately prior to each experiment.

**Salmonella Mutagenesis Assay.** Plates (Falcon no. 1028 Muta-Assay dishes) contained 25 ml Vogel-Bonner Medium E (17) supplemented with 0.5% glucose in 1.5% Difco purified agar. Top agar was prepared by adding 10.0 ml of a 0.5 mM L-histidine-0.5 mM-D-biotin solution, 2.5 ml of a 20% glucose solution, and 2.0 ml of a ×50 concentration of Vogel-Bonner Medium E salts to 100 ml of 0.6% agar. *Salmonella typhimurium* strains TA98 and TA100 were obtained from Dr. Bruce N. Ames (Berkeley, Calif.) and were maintained as frozen stocks at -80° as recommended (1). For use, cultures were grown in 50-ml Oxoid Nutrient Broth No. 2 overnight at 37° with moderate shaking (approximately 150 rpm) and then placed on ice. The ampicillin and crystal violet sensitivities of the strains were checked at the time of each experiment.

**Mutagenicity Experiments with RSV Microsomal Fraction.** Incubation mixtures consisted of 0.5 ml RSV microsomal fraction (diluted to 4 mg protein per ml in 0.15 M KCl, pH 7.4), 0.1 ml bacterial cell culture (approximately 10<sup>8</sup> cells) and the test compound (in 50 μl dimethyl sulfoxide). All concentrations of the test compounds were tested in triplicate. After an initial preincubation in a rotating shaker (150 rpm) for 3 min at 37°, the incubation tubes were removed to ice, and 10 μl arachidonic acid in 95% ethanol were added (final concentration, 100 μM). After mixing, the tubes were returned to the 37° water bath and incubated with shaking for an additional 30 min. Incubation tubes were then kept on ice until the contents were plated. After plating, the top agar was allowed to solidify for up to 30 min and the plates were incubated at 37° for 48 hr, after which the histidine revertant colonies were counted manually. All plates were examined under magnification for evidence of toxicity.

In experiments with indomethacin, the microsomal fraction was preincubated for 3 min at 37° with indomethacin (5 μl of a solution in 95% ethanol to yield a final concentration in the incubation tube of 100 μM) before the addition of bacterial cells and test chemicals. All subsequent steps were as described above.

**Mutagenicity Experiments with Aroclor-Induced Rat Liver S-9.** Incubations with rat liver S-9 were run concurrently with RSV microsomal incubations using the same test chemical stock solutions. The 0.5 ml of RSV microsomal fraction was replaced by 0.5 ml of a standard S-9 mix containing 50 μl of S-9 fraction (approximately 4 mg protein), glucose 6-phosphate, and NADP<sup>+</sup> (1). The preincubation manipulations were the same as for the assays containing RSV microsomal fraction.

## RESULTS

The ability of PGS to cooxidize BP, BA, chrysene, and several of their dihydrodiol metabolites to products mutagenic to *Salmonella* strains TA98 and TA100 was tested. The activation of these compounds by a cytochrome P-450 system present in the rat liver S-9 activation system commonly used in the Ames *Salmonella* assay (1) was also measured.

Initial experiments with an activation system containing a Tween 20-solubilized enzyme preparation from RSV microsomes were unsuccessful because of toxicity to the *Salmonella* (results not shown). Subsequent experiments were therefore performed with RSV microsomal fraction in the absence of detergent.

As seen in Chart 1, the activation system using RSV microsomes (a known source of PGS) with arachidonic acid metabolized BP-7,8-diol to products mutagenic to strain TA98. The

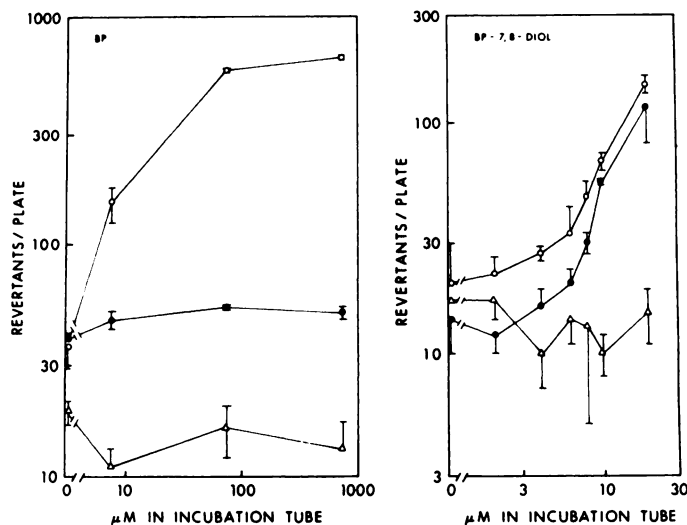


Chart 1. Mutagenicity of BP and BP-7,8-diol with *S. typhimurium* TA98. Experimental details are given in "Materials and Methods." Values are the mean of triplicate plates. ○, rat liver S-9; ●, RSV microsomal fraction; △, control (without enzyme). Bars, S.D.

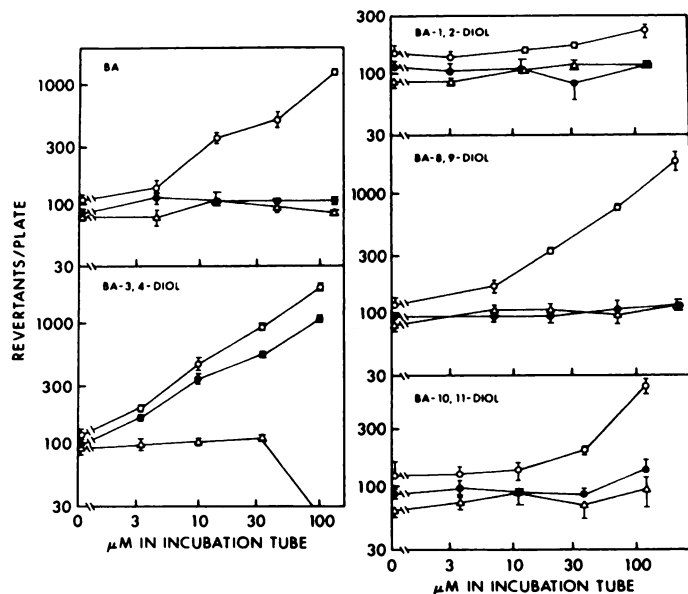


Chart 2. Mutagenicity of BA and BA metabolites with *S. typhimurium* TA100. Experimental details are given in "Materials and Methods." Values are the mean of triplicate plates. ○, rat liver S-9; ●, RSV microsomal fraction; △, control (without enzyme). Bars, S.D.

parent compound, BP, was inactive in this system (at concentrations up to 150 μM). The Aroclor-induced rat liver S-9 (cytochrome P-450) system activated both BP and its 7,8-dihydrodiol derivative.

BA (up to 140 μM) was not activated by RSV microsomes and, of the 4 derivatives tested (BA-1,2-diol, BA-3,4-diol, BA-8,9-diol, and BA-10,11-diol), only BA-3,4-diol was activated to a mutagen by this system (Chart 2). The rat liver S-9 activated BA and all 4 of the BA dihydrodiols tested to metabolites mutagenic to TA100.

The RSV microsomal system did not activate chrysene and, of the 4 chrysene metabolites (chrysene-*trans*-1,2-diol, chrysene-*trans*-3,4-diol, chrysene-*trans*-5,6-diol, and chrysene-*cis*-5,6-diol), only the chrysene-*trans*-1,2-diol was converted

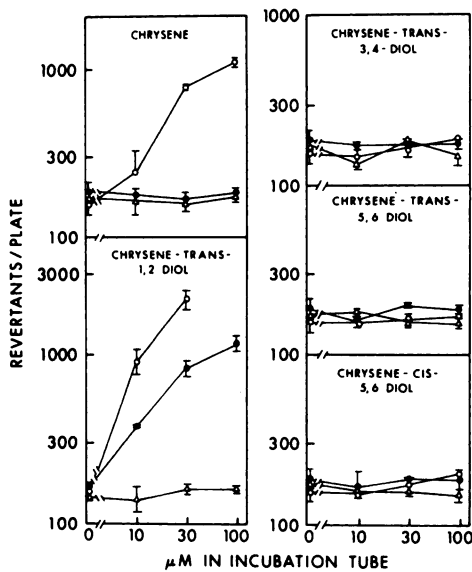


Chart 3. Mutagenicity of chrysene and its metabolites with *S. typhimurium* TA100. Experimental details are given in "Materials and Methods." Values are the mean of triplicate plates. ○, rat liver S-9; ●, RSV microsomal fraction; △, control (without enzyme). Bars, S.D.

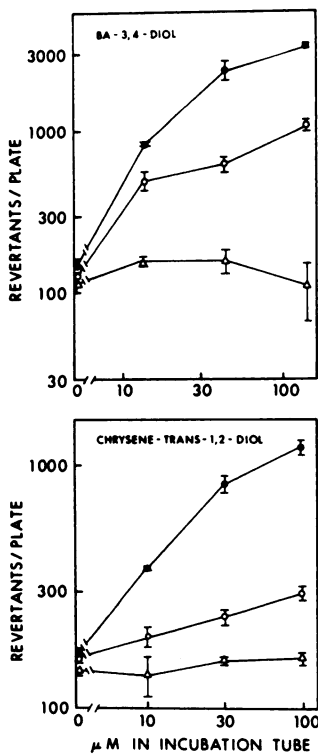
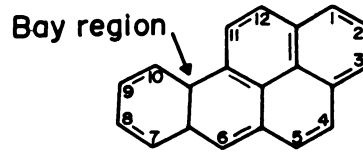


Chart 4. Mutagenicity of BA-3,4-diol and chrysene-*trans*-1,2-diol with *S. typhimurium* TA100. Values are the mean of triplicate plates. Experimental details are given in "Materials and Methods." ●, RSV microsomal fraction; ○, RSV microsomal fraction plus indomethacin; △, control (without enzyme). Bars, S.D.

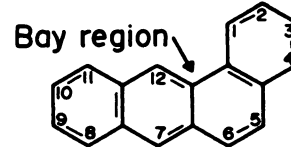
by this system to products mutagenic to TA100 (Chart 3). The rat liver S-9 activated both chrysene and its *trans*-1,2-diol, although no mutagenicity was detected in this system with the other 3 chrysene derivatives.

To confirm that the activation by RSV microsomes was mediated by PGS, the RSV microsomes were incubated with indomethacin, an inhibitor of PGS. As shown in Chart 4, the

Benzo[*a*] pyrene (BP)



Benzo[*a*]anthracene (BA)



Chrysene

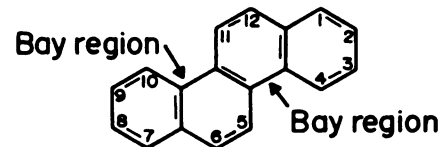


Chart 5. Chemical structure of parent PAH used, showing the "bay regions."

number of histidine revertants produced by incubating BA-3,4-diol or chrysene-1,2-diol with RSV microsomes was reduced by the addition of indomethacin.

DISCUSSION

We have studied the activation of BP, chrysene, BA, and a number of their dihydrodiol metabolites by PGS to products mutagenic to *S. typhimurium* strains TA98 and TA100. Microsomes from RSV, a source of PGS, in the presence of the PGS substrate arachidonic acid, activated only those PAH dihydrodiols containing adjacent bay-region double bonds (BP-7,8-diol, BA-3,4-diol, and chrysene-1,2-diol; see Chart 5). The conversion to mutagenic products was inhibited by indomethacin, an inhibitor of PGS. This supports the hypothesis that these dihydrodiols are cooxidized by PGS. It should also be noted that RSV microsomal fraction contains no detectable cytochrome P-450 (results not shown) and does not support NADPH-dependent oxidation of BP-7,8-diol (5).

To serve as a positive control, the rat liver S-9 activation system commonly used in the Ames *Salmonella* assay was run concurrently with the RSV microsomal assay. Although no quantitative comparison between these 2 activation systems can be made (due to the multiplicity of activating enzymes in liver S-9 and the possibility of inactivation by enzymes in this fraction), a qualitative comparison between the 2 systems is possible. In contrast to the activation by RSV microsomes of only those dihydrodiol precursors of the bay-region diol-epoxides, the rat liver S-9 was able to convert the parent PAH, the dihydrodiol precursors of the bay-region diol-epoxides, and some other dihydrodiols (BA-1,2-diol, BA-8,9-diol, and BA-10,11-diol) to mutagenic products. Thus, the PGS system

appears to be more selective than does the P-450 system in the conversion of the PAH and their metabolites to mutagenic products.

The nature of the mutagenic metabolites formed by PGS from these dihydrodiols is not known. Evidence suggests that BP-7,8-diol is converted to diol-epoxide I by PGS since the metabolite isolated from incubation mixtures was 7,10/8,9-tetrol (5, 15) and the polyguanylate adduct formed in incubations of BP-7,8-diol with PGS was the diol-epoxide I adduct (12). Chrysene-1,2-diol and BA-3,4-diol may be similarly converted to the corresponding bay-region diol-epoxides by PGS.

The biological relevance of PGS in the activation of select PAH dihydrodiols is unknown. The parent compound must first be converted to the appropriate dihydrodiol by the P-450 system before cooxidation by PGS to mutagenic metabolites. PGS is ubiquitous throughout mammalian tissues and occurs at significant levels in organs which are targets for PAH-induced cancers (lung, gut, kidney, and skin). Recent evidence demonstrates the ability of pulmonary tissues, including human lung, to metabolize BP-7,8-diol in the presence of the arachidonic acid substrate (14). Thus, it is possible that PGS is a significant alternative means of formation of bay-region diol-epoxides, the hypothesized ultimate mutagenic and carcinogenic metabolite of PAH.

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