1-Ethynylpyrene, a Suicide Inhibitor of Cytochrome P-450 Dependent Benzo[*a*]pyrene Hydroxylase Activity in Liver Microsomes†

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ABSTRACT: The preparation of 1-ethynylpyrene (EP) is described. Incubation of EP with liver microsomes in the presence of NADPH yields fluorescent products, but the production of these products ceases after 15 min. Addition of fresh microsomes restores the original rate of EP metabolism. The metabolism of EP is initially more rapid in microsomes from 5,6-benzoflavone- (BF) pretreated rats than in microsomes from phenobarbital- (PB) pretreated rats or from untreated, control animals. EP strongly inhibits the hydroxylation of benzo[*a*]pyrene (BP) by liver microsomes; after 20 min in the presence of EP, BP metabolism nearly ceases. Addition of fresh microsomes restores the original rate of BP hydroxylation. EP more effectively inhibits the oxidation of BP in liver microsomes from rats pretreated with BF than from rats pretreated with PB or from untreated, control animals. The inhibition of BP hydroxylation activity due to EP is dependent upon NADPH and is apparently irreversible. Kinetic analyses demonstrate that the observed inhibition of BP hydroxylation is due to loss of the enzymatic activity by a process that is first order in EP and that reaches a limiting value at infinite EP concentrations. One such first-order process, with a $t_{1/2}$ of 3.5 min and a K_s for EP of 40 μM, is observed in microsomes from BF-pretreated rats. Two such first-order processes, one with $t_{1/2}$ of 6.9 min and K_s of 46 μM and one with $t_{1/2}$ of 12.7 min and K_s of 33 μM, are observed in microsomes from PB-pretreated rats. It is proposed that a self-catalyzed inhibition (suicide inhibition) of the cytochrome P-450 dependent BP hydroxylation occurs in the presence of EP. Incubation with EP under conditions that result in loss of about 90% of the BP hydroxylase activity in microsomes from BF-pretreated rats and about 66% of the activity in microsomes from PB-pretreated rats causes the loss of only 6 and 12% of the cytochrome P-450, respectively. It is concluded that loss of P-450 content is an insensitive measure of the effect of this inhibitor upon this cytochrome P-450 dependent enzyme activity. The selectivity of the loss of P-450 due to the incubation of the different microsomal preparations with EP is also observed to be different than the selectivity for loss of BP hydroxylase activity. It is proposed that the suicide inhibition of cytochrome P-450 dependent enzymes by alkynes need not involve heme alkylation and a resulting loss of P-450 content. In vivo exposure of rats to EP does not cause a significant change in the cytochrome P-450 content in the liver microsomes subsequently isolated. The in vivo exposure also did not result in the elimination of the BP hydroxylation activity in the liver microsomes.

Recently, Ortiz de Montellano and his associates have established that certain alkynes cause a self-catalyzed destruction (suicide destruction) of the cytochrome P-450 content of cytochrome P-450 dependent monooxygenases [Ortiz de Montellano & Kunze, 1980a,b, 1981a; Ortiz de Montellano et al., 1982; see also Ortiz de Montellano & Correia (1983) for a review]. These workers have demonstrated that the reactions of selected alkynes which are catalyzed by monooxygenases lead to significant loss of the cytochrome P-450 content of hepatic microsomes; in some cases, new alkylated heme moieties derived from covalent attachments of the alkyne substrates have been isolated and identified. It has been proposed that additions of the activated oxygen of the cytochrome oxene–iron complexes to the carbon–carbon triple bonds create reactive intermediates that can alkylate the heme nitrogen atoms and thereby destroy the prosthetic group of the cytochrome P-450 dependent enzymes. According to Ortiz de Montellano and Kunze, the heme alkylations and resulting P-450 destructions observed with alkynes represent side reactions of the intermediates produced from these substrates during the conversion sequence normally catalyzed by P-450-dependent monooxygenases (Ortiz de Montellano & Kunze, 1980a, 1982).

In a separate area of study, Jerina et al. (1982) proposed a model for the substrate binding site of the cytochrome P-450$_c$[1] dependent monooxygenase of rat liver. This model is based upon the observed stereoselective formation of arene oxides from several aryl hydrocarbons by cytochrome P-450$_c$ containing monooxygenase systems [see also van Bladeren et al. (1982)]. It postulates a cytochrome P-450$_c$ catalytic site with a template region that binds potential aryl hydrocarbon substrates in fixed orientations relative to the location of the active oxene–iron complex.

If the two features of cytochrome P-450 dependent monooxygenase catalysis described above could be combined in a single substrate molecule, a new type of selective inhibitor of monooxygenase activity should result. Since cytochrome P-450$_c$, the major P-450 monooxygenase found in the liver of rats treated with 3-methylcholanthrene (Thomas et al., 1979, 1981), displays a higher specific activity with carcinogenic aryl hydrocarbon substrates such as BP than do the other characterized forms of cytochrome P-450 (Ryan et al., 1980), a substrate that could selectively cause the self-catalyzed inactivation of cytochrome P-450$_c$ would be an interesting experimental tool. Such a substrate might also have some therapeutic uses in aryl hydrocarbon carcinogenesis.

In an initial attempt to test the feasibility of combining the features of cytochrome P-450 activity described above, we

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[1] Abbreviations: P-450$_c$, the major species of cytochrome P-450 induced in rat liver by 3-methylcholanthrene [see Lu & West (1980) and Guengerich et al. (1982)]; BP, benzo[*a*]pyrene; EP, 1-ethynylpyrene; BF, 5,6-benzoflavone; PB, phenobarbital; GP, glucose 6-phosphate; GPDH, glucose-6-phosphate dehydrogenase; EDTA, ethylenediaminetetraacetic acid.

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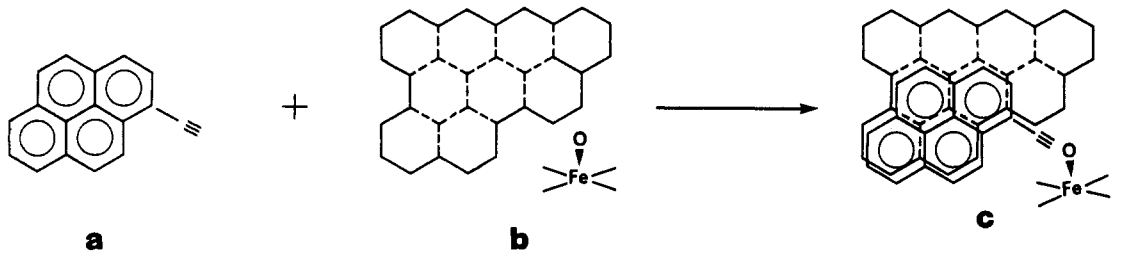

FIGURE 1: (a) Structure of EP; (b) proposed substrate site of P-450$_c$ showing active Fe–oxene below plane of binding domain; (c) EP bound to P-450$_c$ site in a possible orientation that would place the triple bond proximate to the Fe-activated oxygen atom.

prepared EP. Figure 1 illustrates one possible orientation of this substrate within the proposed catalytic region of cytochrome P-450$_c$. This figure indicates how association of the pyrene portion of the molecule with the aryl hydrocarbon template postulated by Jerina et al. (1982) could position the carbon–carbon triple bond in that region of the catalytic site postulated to contain the oxene–iron complex. This ethynyl group will therefore be suitably oriented to participate in a suicide destruction reaction as proposed by Ortiz de Montellano and his associates for potential alkynyl substrates of the P-450-dependent monooxygenases.

The effects of EP upon monooxygenase activities in microsomal preparations from rat liver that are described in this paper indicate that this compound reacts in vitro as predicted from the hypotheses outlined above. We find that EP is a substrate for the P-450-dependent microsomal monooxygenases, that NADPH-dependent metabolism of this alkyne substrate results in the self-catalyzed, irreversible inactivation of monooxygenase activity, and that these properties of EP are preferentially manifest in microsomal preparations that contain higher levels of cytochrome P-450$_c$ (preparations from rats pretreated with BF).

Materials and Methods

Chemicals. Before use, tetrahydrofuran was distilled from sodium benzophenone dianion in a nitrogen atmosphere and toluene was distilled from sodium metal in a nitrogen atmosphere. Other solvents were reagent grade and were used without further purification. The reagents pyrene, *N*-methylformanilide, *o*-dichlorobenzene, triphenylphosphine, dibromomethane, BP, and BF were obtained from Aldrich Chemical Co., phosphorus oxychloride was obtained from MCB Manufacturing Chemists, PB, NADP, NADPH, GP, and GPDH were from Sigma Chemical Co., 4-phenyl-1-butyne was from Farchan Division, Story Chemical Co., Bio-Rad protein reagent was from Bio-Rad Laboratories, and [^{3}H]-benzo[*a*]pyrene (purity 98.6%, 20 Ci/mmol) was obtained from Amersham/Searle and diluted with unlabeled BP to 16.7 mCi/mmol.

Synthesis and Identification of EP. EP was prepared by a Vilsmeier's reaction (Vollmann et al., 1937) and a modified Wittig reaction according to the procedures described by Tanikawa et al. (1968) and by Matsumoto & Kuroda (1980). Thin-layer chromatography was performed on Merck glass-backed silica gel 60 plates (0.25-mm thickness, F-254) with petroleum ether as solvent. Compounds were visualized both with ultraviolet light and with iodine vapor. The technique of flash chromatography as described by Still et al. (1978) was used to purify the EP product. Silica gel 60, 230–400 mesh, from Merck & Co., Inc., was used with petroleum ether as the eluting solvent. Melting point determinations were obtained with a Thomas capillary melting point apparatus. Ultraviolet spectra were obtained with a Beckman 3600 spectrophotometer, and infrared spectra were obtained on a Perkin-Elmer 683 spectrophotometer. ^{1}H nuclear magnetic resonance spectra were obtained with a Varian EM 390 spectrometer in deuteriochloroform as solvent, and the chemical shifts are reported in parts per million downfield from internal tetramethylsilane. Carbon-13 nuclear magnetic resonance spectra (proton decoupled) were obtained on a JEOL JNM-FX 60 spectrometer at an operating frequency of 15.03 MHz in deuteriochloroform as solvent, and the chemical shifts are reported in parts per million relative to $CDCl_3$ as an internal standard (77.18 ppm).

The formylation of pyrene with *N*-methylformanilide and phosphorus oxychloride in *o*-dichlorobenzene afforded crystalline 1-formylpyrene in 50% yield: mp 126–127 °C (ethanol). The reaction of 1-formylpyrene with (bromomethyl)-triphenylphosphonium bromide and 3 equiv of potassium *tert*-butoxide in tetrahydrofuran gave EP. The EP was separated from some 1-(2-bromovinyl)pyrene intermediate by flash column chromatography. The product fraction, which gave a single spot upon thin-layer chromatography (silica gel with petroleum ether), gave crystalline EP (petroleum ether) in 63% yield: mp 105–106 °C; IR (neat) 3280 (s, ≡CH), 3020 (m, aromatic H), 2080 (w, C≡C), 1580 (w, aromatic ring) cm^{-1}; UV 352 nm (s), 340 (m), 280 (s), 270 (m), 245 (s), 235 (m); ^{1}H NMR ($CDCl_3$) δ 3.56 (s, 1 H, CH), 7.62–8.51 (m, 9 H, $C_{16}H_9$); ^{13}C NMR ($CDCl_3$) δ 73.5 (≡C-H), 82.7 (—C≡), 121.5–127 ($C_{16}H_9$).

Microsome Preparation. Young male Sprague-Dawley rats (90–150 g) were injected intraperitoneally with 50 mg (0.184 mmol) of BF dispensed in 0.6 mL of corn oil (Mazola) per kg of body weight or with 41 mg (0.184 mmol) of EP in corn oil per kg of body weight on 3 successive days and then sacrificed on the fourth day. Animals injected with corn oil alone served as the control animals. Separate groups of animals were provided drinking water containing 0.1% PB for four successive days. Microsomal preparations were obtained according to the procedures described by Ganu & Alworth (1978) and by Viswanathan & Alworth (1981); the microsomes were then washed twice with isotonic KCl according to the procedure of Omura & Sato (1964).

Assay Procedures. The protein concentration of the microsomal preparations was determined by the method of Bradford (1976). Microsomal proteins were added in 0.1 mL of 0.1 M tris(hydroxymethyl)aminomethane (Tris) buffer, pH 7.4, per 5 mL of diluted Bio-Rad reagent. The absorbance maxima at 595 nm were measured with a Bausch & Lomb Spectronic 20 spectrophotometer. Bovine serum albumin was used as a protein standard.

The standard fluorometric assay for BP and EP hydroxylation was performed essentially as described by Nebert & Gelboin (1968). A NADPH-regenerating system containing NADP (0.5 mM), GP (5 mM), and GPDH (0.36 unit/mL) was used instead of NADPH. Solutions of BP and EP in acetonitrile were prepared and aliquots of 25 μL added per 1 mL of assay medium to yield 80 μM substrate concentra-

tions. Microsomal protein concentrations were 0.25 and 0.60 mg/mL. The fluorescence was measured with a Perkin-Elmer 650-10S instrument by using slit widths of 1.5 nm and excitation and emission wavelengths of 396 and 522 nm for BP and of 430 and 498 nm for EP, respectively. All determinations were carried out in duplicate; comparable results were obtained with different microsomal preparations.

A radiochemical assay using ^{3}H-labeled BP, based upon the assay developed by Nesnow et al. (1977), was also used to measure the cytochrome P-450 dependent hydroxylation of BP. The concentrations of EP were varied from 0.6 to 120 μM in the presence of a constant 60 μM BP concentration. The EP and BP were added as acetonitrile solutions; 50 μL of acetonitrile was added per 1 mL of assay medium. Microsomal protein concentration was varied from 0.10 to 0.60 mg/mL. A 300-μL aliquot of the final alcoholic phase was added to 10 mL of Handifluor (Mallinckrodt) scintillation solution, and the radioactivity was measured in a Beckman LS 7000 liquid scintillation counter with internal standardizations.

Cytochrome P-450 concentrations were determined on a Beckman 3600 spectrophotometer by measuring the difference in absorption at 450 nm between a dithionite-reduced, carbon monoxide treated sample and a dithionite-reduced, carbon monoxide untreated reference sample, according to the procedure of Omura & Sato (1964). The concentration of microsomal protein was 1 mg/mL. Cytochrome P-450 inactivation was assayed essentially according to the procedure described by Ortiz de Montellano & Mico (1981b). Modifications were the use of a NADPH-regenerating system consisting of NADP (1 mM), GP (4.5 mM), $MgCl_2$ (3 mM), and GPDH (0.36 unit/mL) in the assay medium. EP and 4-phenyl-1-butyne were added in 10 μL of acetonitrile/mL of assay medium to produce concentrations of 120 μM and 1 mM, respectively. After a 3-min preincubation period at 37 °C, the assays were initiated by addition of the NADPH-regenerating system to the rest of the mixture. Aliquots of 1.5 mL were withdrawn and transferred to semimicrospectrometer cells immersed in an ice bath immediately (zero time) and after 20 min. Each set of experiments included measurements of control samples—incubations containing acetonitrile but no added alkyne. At least two measurements with different microsomal preparations in the presence and in the absence of EP or 4-phenyl-1-butyne were performed.

Gel Filtration Chromatography. Microsomes from BF-pretreated rats were incubated with 60 μM EP in the presence of NADPH for 30 min at 37 °C, and the resulting reaction mixture was centrifuged at 10000*g* for 40 min. The resulting pellet was resuspended in a minimal volume (~0.5 mL) of 50 mM tris(hydroxymethyl)aminomethane buffer, pH 7.5, and applied to a 1 × 13 cm column of Sephadex G-25. The protein-containing fraction (280 nm) that eluted from the Sephadex column was analyzed with a Hewlett-Packard 8451A spectrometer and a SPEC IIIc spectrofluorometer, extracted with 1.5 volumes of hexane, and then reanalyzed. The hexane extract was extracted with 1 N NaOH, and the UV–vis and fluorescent spectra of the resulting hexane and aqueous base fractions were recorded. In a control experiment, microsomes were mixed with 60 μM EP in the absence of NADPH and then subjected to the same fractionation and spectral analysis.

Results

Figure 2 illustrates the production of fluorescent products, as determined by the assay procedure of Nebert & Gelboin (1968), when BP (○) or EP (□) is incubated with washed liver microsomes from rats pretreated with PB (Figure 2a) or with BF (Figure 2b). It was established that the production of these fluorescent metabolites requires the presence of NADPH (data not shown); as illustrated, the formation of fluorescent metabolites from both BP and EP is more rapid and more extensive with microsomes from rats pretreated with BF. The data plotted in Figure 2 emphasize a significant difference between the microsomal-dependent metabolism of BP and EP. Under the experimental conditions (80 μM substrate, 250 μg of microsomal protein/mL), the production of fluorescent metabolites from BP by both types of microsomes is linear for at least 15 min and proceeds at a measurable rate for at least 35 min. The production of fluorescent metabolites from EP, however, decreases rapidly; after 15 min, only a fraction of the original activity remains. This effect is apparent with microsomes from animals pretreated with BF or PB. (Microsomes from control animals produce comparable results; data not shown.) When less microsomal protein is present in the incubation, the total yield of fluorescent EP metabolites is decreased but production of these metabolites still declines rapidly; adding NADPH after 20 min does not cause the

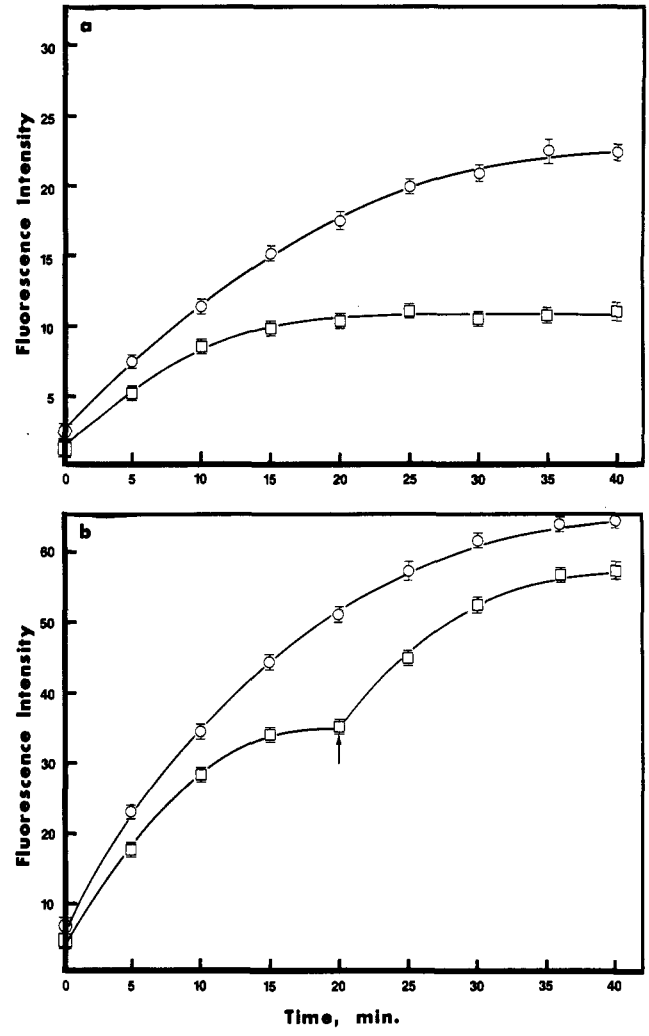

FIGURE 2: Time course for the formation of fluorescent metabolites from BP (○) and EP (□). The incubations were conducted at 37 °C in 50 mM Tris buffer, pH 7.5, containing 0.5 mM NADP, 5 mM GP, 0.36 unit/mL GPDH, 3 mM $MgCl_2$, and 80 μM substrate. The fluorescence was measured by using 1.5-nm slit widths and excitation and emission wavelengths of 396 and 522 nm for BP and of 430 and 498 nm for EP, respectively. (a) Incubation with 250 μg/mL microsomal protein from PB-pretreated rats; (b) incubation with 250 μg/mL microsomal protein from BF-pretreated rats. The arrow indicates the addition of fresh microsomal protein (250 μg/mL) from BF-pretreated rats.

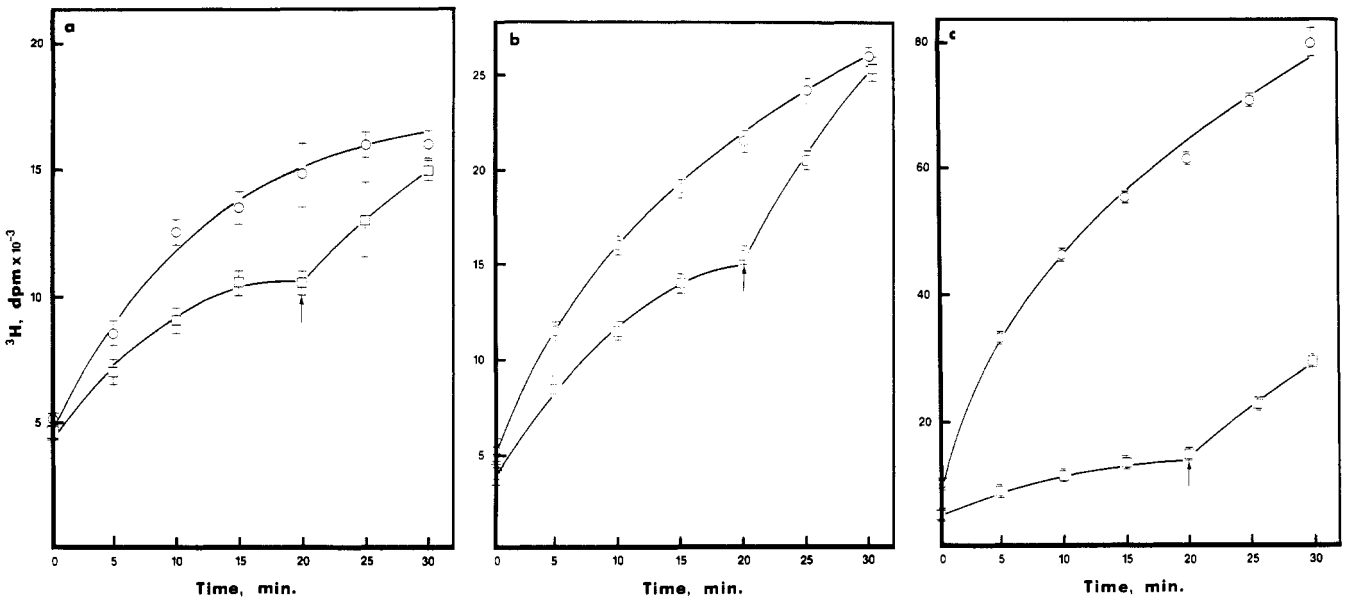

FIGURE 3: Time course for the conversion of [^{3}H]BP into polar radioactive metabolites in the presence (□) and the absence (○) of EP. (a) Incubation with 614 μg/mL microsomal protein from control rats; (b) incubation with 592 μg/mL microsomal protein from rats pretreated with PB; (c) incubation with 596 μg/mL microsomal protein from rats pretreated with BF. The incubations were conducted at 37 °C in 50 mM phosphate buffer, pH 7.5, containing 1 mM NADP, 4.5 mM GP, 3 mM $MgCl_2$, 0.36 unit/mL GPDH, and 60 μM BP. EP, when added (□), was also present at 60 μM. The arrows indicate addition of fresh microsomal protein to each assay mixture: (a) 885, (b) 888, and (c) 859 μg/mL.

production of additional fluorescent metabolites. As illustrated in Figure 2b, however, the addition of fresh microsomes to the EP incubation after 20 min restores the production of fluorescent metabolites to a rate that is comparable to that originally measured.

Figure 3 illustrates the effect of unlabeled EP upon the production of radioactive metabolites from ^{3}H-labeled BP by liver microsomes from control animals (Figure 3a) and from rats pretreated with PB (Figure 3b) or BF (Figure 3c), as measured by the BP hydroxylation assay of Nesnow et al. (1977). The data plotted compare the rates of production of metabolites from 60 μM BP in the absence of EP (○) and in the presence of 60 μM EP (□). The data shown in Figure 3 are consistent with those shown in Figure 2 and demonstrate that liver microsomes from rats exposed to BF are significantly more effective at catalyzing the metabolism of BP than are liver microsomes from control or PB-exposed animals. (Note changes in the ordinate scales.) In each case, however, the addition of EP to the incubation mixture markedly inhibits the observed BP hydroxylation; when coincubated with 60 μM EP, the metabolism of 60 μM BP essentially ceases after 15–20 min while, under the same experimental conditions, the metabolism of BP in the absence of EP continues at a significant rate for 30 min. As previously illustrated for the metabolism of EP (Figure 2b), Figure 3 shows that addition of fresh microsomal protein to the incubations that are strongly inhibited by EP restores the BP metabolism to its original rate.

Microsomes (5 mg/mL) were also preincubated at 37 °C with 60 μM EP for 10 min in the presence or in the absence of NADPH, the incubation solutions were diluted 10-fold, BP (60 μM) was added, and the resulting rates of BP hydroxylation were then determined. In Figure 4, the resulting rates of BP hydroxylation are compared with the rates observed when the microsomes (5 mg/mL) were preincubated at 37 °C in the absence of EP and diluted 10-fold, BP (60 μM) was added, and the BP hydroxylation was then measured in the presence of 6.0 μM EP. The data plotted in Figure 4 illustrate that the rate of BP hydroxylation is decreased in the presence of 6.0 μM EP. The same decreased rates, however, were

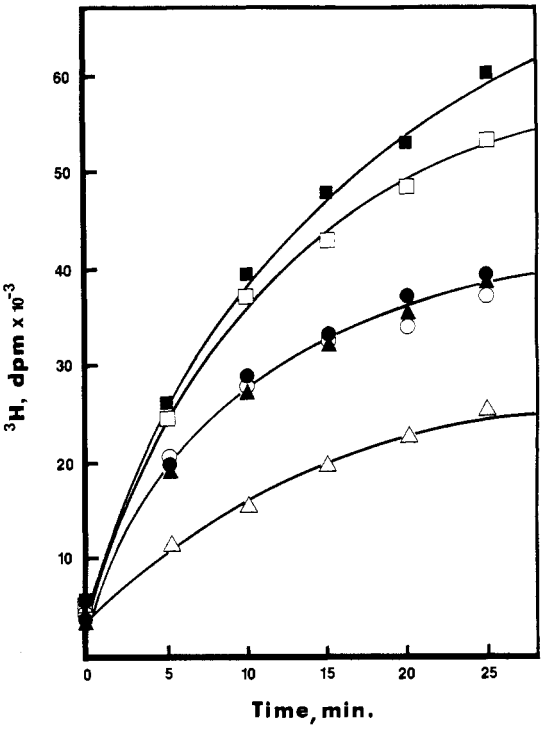

FIGURE 4: Time course for the conversion of [^{3}H]BP into polar radioactive metabolites. The assays were conducted at 37 °C in 50 μM phosphate buffer, pH 7.5, containing 3 mM $MgCl_2$, 1 mM NADPH, and 60 μM [^{3}H]BP. The microsomes used in these assays, from rats pretreated with BF, were preincubated at a concentration of 5 mg/mL for 10 min at 37 °C in 50 μM phosphate buffer, pH 7.5, containing 3 mM $MgCl_2$, the preincubation solution was diluted 10-fold, and then the assay of BP hydroxylation activity was immediately initiated. (■) Preincubation in the absence of EP and of NADPH; assay in the absence of EP. (□) Preincubation with 1 mM NADPH in the absence of EP; assay in the absence of EP. (●) Preincubation in the absence of EP and of NADPH; assay in the presence of 6.0 μM EP. (○) Preincubation with 1 mM NADPH in the absence of EP; assay in the presence of 6.0 μM EP. (▲) Preincubation with 60 μM EP in the absence of NADPH; assay in the presence of 6.0 μM EP. (△) Preincubation with 60 μM EP and 1 mM NADPH; assay in the presence of 6.0 μM EP.

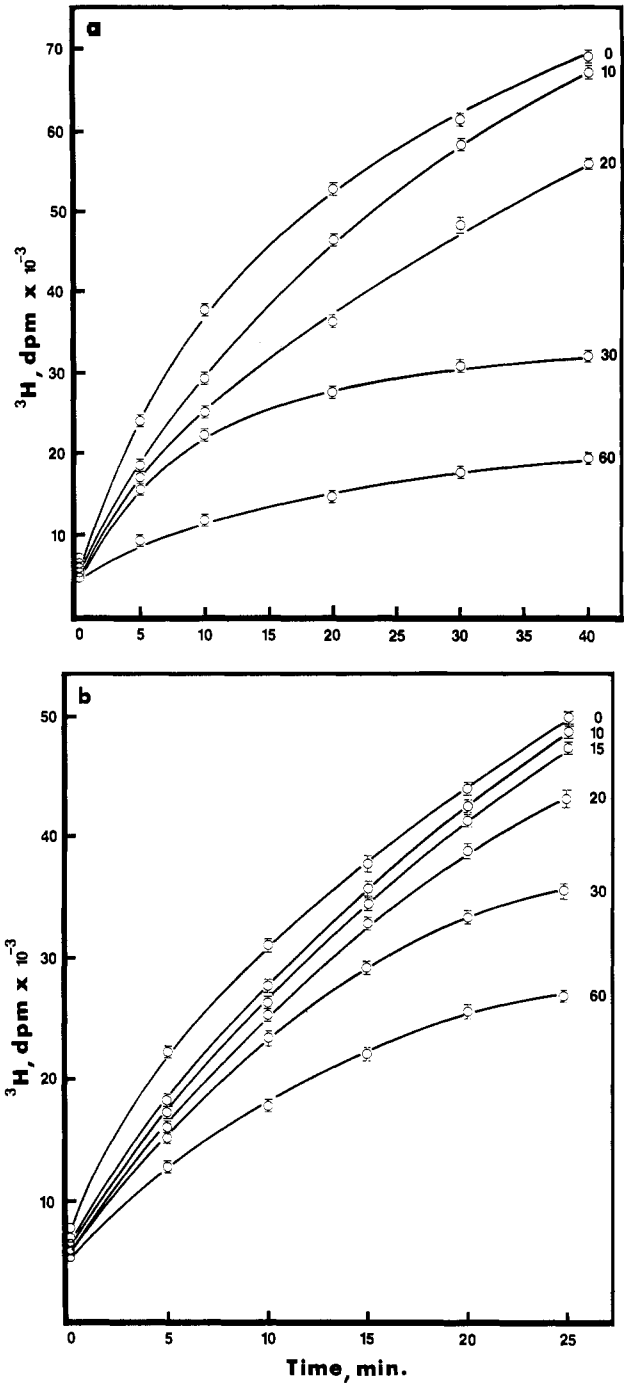

FIGURE 5: Time course for the conversion of [^{3}H]BP into polar radioactive metabolites in the absence and presence of varying EP concentration. The concentrations of EP (μM) are indicated in the figure for each experimental curve. (a) Incubation with 400 μg/mL microsomal protein from rats pretreated with BF; (b) Incubation with 400 μg/mL microsomal protein from rats pretreated with PB. Assay conditions are as described in Figure 3.

observed whether the microsomes had been preincubated in the presence of 60 μM EP but in the absence of NADPH or in the presence of NADPH but in the absence of EP. In contrast, when the microsomes were preincubated in the presence of 60 μM EP and NADPH and diluted 10-fold and the rate of BP hydroxylation was then assayed in the presence of the resulting 6.0 μM EP, the measured rates were significantly lower than the other rates measured in the presence of 6.0 μM EP (Figure 4). The addition of fresh microsomes to this latter incubation after 25 min of BP hydroxylation restored the rate of BP hydroxylation to that observed in the control incubation that lacked EP (data not shown). The

Table I: Kinetic Parameters for the Inhibition of BP Hydroxylation by EP

	BF microsomes			PB microsomes	
	coincubation with BP[a]	20-min preincubation[b]	combined incubation data[c]	coincubation with BP[d]	20-min preincubation[e]
$t_{1/2}$ (min)[f]	3.9	3.6	3.5	6.9	12.7
k (10^{-3} s^{-1})[g]	3.0	3.2	3.2	1.7	0.9
K_s (μM)[h]	35	39	40	46	33

[a] Figure 7a (○). [b] Figure 7a (□). [c] Figure 7a (○ and □). [d] Figure 7b (○). [e] Figure 7b (□). [f] The time required to inactivate half of the hydroxylase activity at infinite EP concentration. [g] First-order rate constant for the observed inactivation. [h] Apparent dissociation constant of EP enzyme complex.

results summarized in Figure 4 thus indicate that the loss of BP hydroxylation activity observed in the presence of EP is dependent upon NADPH and is also irreversible.

Figure 5 illustrates the inhibitory effect of varying EP concentrations upon the production of radioactive metabolites from 60 μM ^{3}H-labeled BP by liver microsomes from rats pretreated with BF (Figure 5a) or PB (Figure 5b). Comparison of the data in Figure 5 again establishes that the hydroxylation of BP is more strongly inhibited by EP in microsomal preparations from rats exposed to BF than it is in microsomal preparations from PB-exposed animals. (Note the ordinate scales.)

Analyses of the type of kinetic data plotted in Figure 5 are shown graphically in Figures 6 and 7. Two types of experiments were performed to measure the rate of loss of BP hydroxylation activity in the presence of EP. In one set of experiments the decreased rates of BP hydroxylation in the presence of variable EP concentrations were established by coincubating the EP with 60 μM BP (Figure 5). A polynomial expression that produced the best nonlinear least-squares fit with the experimental data was then determined by using a Hewlett-Packard 9826 microcomputer. The log values of the activities were then calculated from the determined polynomial expression, and a linear least-squares fit of these log values vs. incubation times was calculated. These data are plotted in Figure 6a,b. The time required for loss of 50% of the initially observed activity ($t_{1/2}$) was thus established for each EP concentration. The $t_{1/2}$ values determined for the measured losses of BP hydroxylase activity are plotted in Figure 7 (○) vs. the reciprocals of the EP concentrations for microsomal preparations from BF-exposed rats (Figure 7a) and for microsomal preparations from PB-exposed rats (Figure 7b). In the second set of experiments, the microsomes were preincubated for 20 min in the presence of NADPH and variable amounts of EP, then BP substrate was added, and the rates of BP hydroxylation were measured during an additional 10-min coincubation period (data not shown). The log values of these measured rates, determined with the aid of a microcomputer as described above, are plotted vs. the BP incubation times in Figure 6c,d. The $t_{1/2}$ values determined from the data plotted in Figure 6c,d for the measured losses of BP hydroxylase activity in this second set of experiments are also plotted in Figure 7 (□). As shown in Figure 7a, in the case of the microsomal preparations from BF-exposed rats, the $t_{1/2}$ values obtained from the two types of experiments lie on a single straight line. Kinetic parameters were calculated from the data points illustrated in Figure 7a by a linear least-squares program, and these parameters are summarized in Table I. In contrast, Figure 7b shows that in microsomal preparations from PB-exposed rats the $t_{1/2}$ values for the two types of experiments described above determine two separate straight

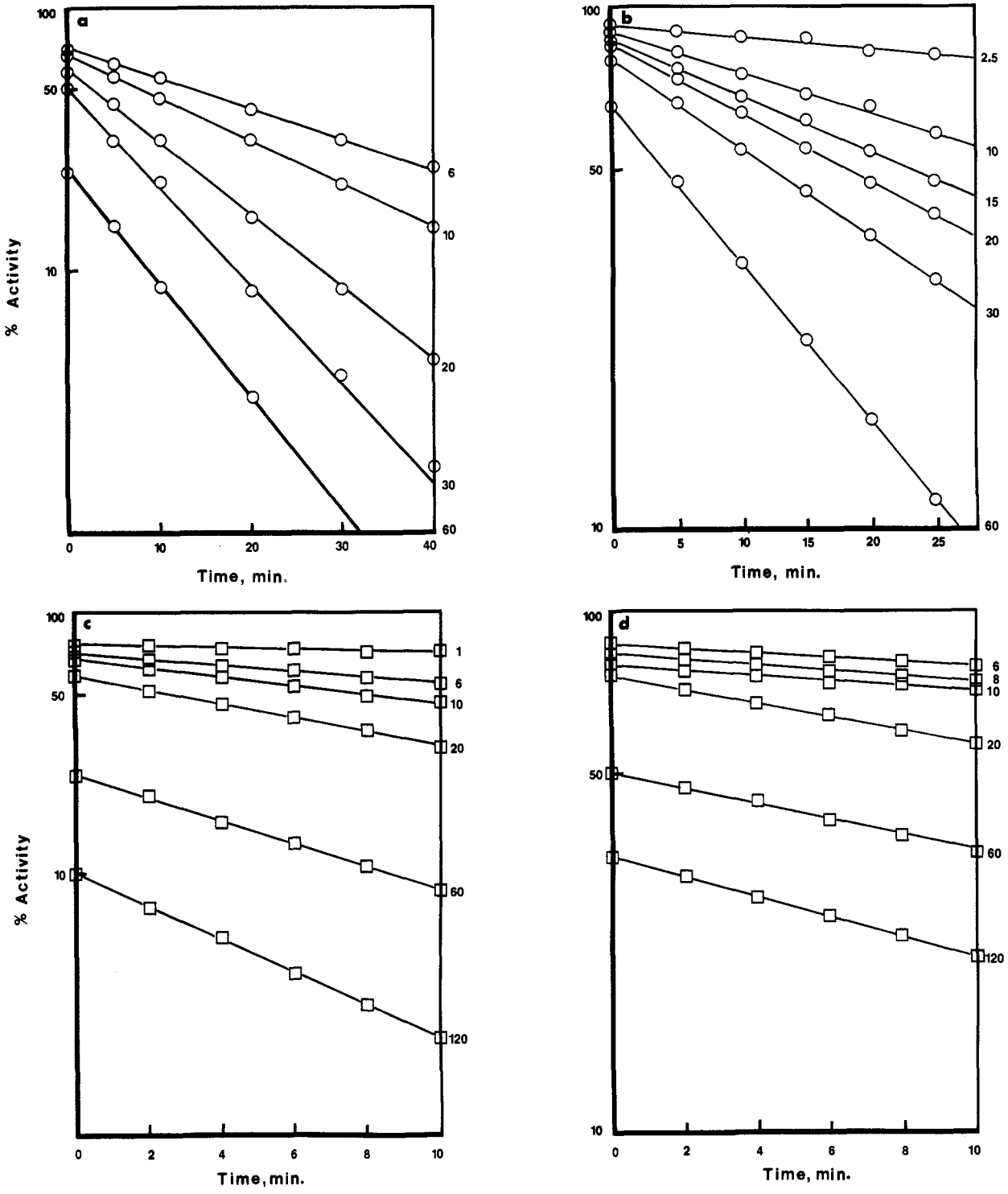

FIGURE 6: Plots of the log values of the percentage of BP hydroxylase activity observed vs. the incubation times. Assay conditions are as described in Figure 3. (a) Incubation of microsomes from BF-pretreated rats in the presence of 60 μM BP and variable concentrations of EP; (b) incubation of microsomes from PB-pretreated rats in the presence of 60 μM BP and variable concentrations of EP; (c) incubation of microsomes from BF-pretreated rats in the presence of 60 μM BP and variable concentrations of EP after the microsomes were preincubated 20 min in the presence of NADPH and the variable EP concentrations; (d) incubation of microsomes from PB-pretreated rats in the presence of 60 μM BP and variable concentrations of EP after the microsomes were preincubated 20 min in the presence of NADPH and the variable EP concentrations. The assays illustrated in (c) and (d) were initiated by the addition of BP to the preincubation mixtures; the variable concentrations of EP used (μM) are indicated on the right side of the figures.

lines, and analysis of the data points in Figure 7b thus yields the two sets of kinetic parameters summarized in Table I. In both sets of experiments, however, the linear plots illustrated in Figures 6 and 7 establish that the observed losses of BP hydroxylase activity are the result of processes that manifest first-order dependence upon EP concentration. The kinetic parameters calculated in Table I also demonstrate that the loss of BP hydroxylation activity in preparations from BF-exposed rats has a higher first-order rate constant and a lower $t_{1/2}$ than either of the first-order losses of activity that occur in preparations from PB-exposed animals.

In addition to the continuing *decrease* in BP hydroxylase activity that has a first-order kinetic dependence upon the EP concentration, it should be noted that the hydroxylation rates determined at zero time indicate that EP also *inhibits* the BP hydroxylase activity initially present in the microsomes in a

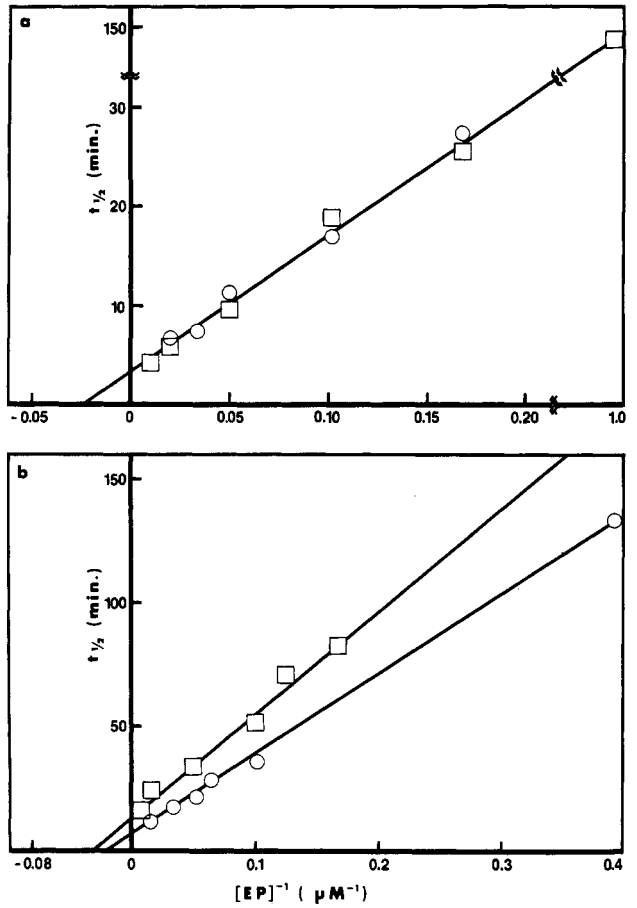

FIGURE 7: Plots of observed $t_{1/2}$ values for the rates of incubation of BP hydroxylase activity vs. the reciprocals of the added EP concentrations. (a) Data from incubations of BP and EP with 600 μg/mL microsomal protein from BF-pretreated rats; (b) data from incubations with 600 μg/mL microsomal protein from PB-pretreated rats. The incubation conditions used in these studies were those described in Figure 3. (○) $t_{1/2}$ values for the inactivation rates determined by coincubations of BP and EP; (□) $t_{1/2}$ values for the inactivation rates determined after 20-min preincubation with EP and NADPH.

Table II: EP Effects on Cytochrome P-450 Content and BP Hydroxylase Activity

		BF microsomes		PB microsomes	
substrate	concn (mM)	BP hydroxylation (% lost)[a]	cyt P-450 (% lost)[b]	BP hydroxylation (% lost)[a]	cyt P-450 (% lost)[b]
EP	0	0	0	0	0
	0.12	90	6	66	12
4-phenyl-1-butyne	0	0	0	0	0
	1.00	62	14	56	27

[a] Activities were measured after 20-min preincubation of alkyne with microsomal protein in the presence of NADPH. Assay conditions are as described in Figure 3. [b] Incubations were conducted at 37 °C for 20 min in 0.1 M phosphate buffer, pH 7.4, containing 1 mg/mL microsomal protein, 1 mM NADP, 4.5 mM GP, 0.36 unit/mL GPDH, 3 mM $MgCl_2$, 1.5 mM EDTA, and 150 mM KCl.

concentration-dependent manner (Figure 6a,b). Both of these effects are consistent with EP functioning as a substrate that can lead to suicide inhibition of the cytochrome P-450 dependent monooxygenase activity.

Table II summarizes the losses of cytochrome P-450 content observed in microsomal preparations from BF-pretreated rats after 20-min incubations in the presence of EP or 4-phenyl-1-butyne. The cytochrome P-450 destructions were measured as described by Ortiz de Montellano & Mico (1981b) using the P-450 assay of Omura & Sata (1964). Table II also shows the decreases in BP hydroxylase activities that occur when

Table III: Cytochrome P-450 Content and Specific Activity of Different Microsomal Samples

pretreatment[a]	P-450 hemoprotein (nmol of heme/mg of protein)[b]	sp act. [^{3}H]BP (pmol mg^{-1} min^{-1})[c]	sp act. EP (FI mg^{-1} min^{-1})[d]
corn oil	0.86	183	12.5
EP	0.97	245	12.3
BF	1.58	891	26.6
PB	2.61	472	16.7

[a] Rats (3–5) were pretreated with indicated chemical on 3 successive days and then sacrificed on day 4 and the livers pooled for analysis. [b] P-450 content was determined in samples that contained 1 mg of microsomal protein/mL of 0.1 M phosphate buffer, pH 7.0. Four measurements on each sample were made; the average values are reported. [c] Microsomal protein concentration was 400 μg/mL; other assay conditions are described in Figure 3. [d] Microsomal protein concentration was 600 μg/mL; EP concentration was 120 μM; other assay conditions are as described in Figure 2. The fluorescence was measured by using 2.0-nm slit widths and excitation and emission wavelengths of 430 and 498 nm, respectively. The fluorescence intensity (FI) reported is the experimental result in arbitrary units.

these two types of microsomal preparations are incubated with the two alkynes.

Concentrations of cytochrome P-450 heme, as established by the method of Omura & Sato (1964), in the liver microsomes from rats exposed in vivo to EP, BF, PB, or corn oil are listed in Table III. Specific activities of the different microsomal preparations with BP and EP as substrates are also listed in Table III.

Gel filtration chromatography was performed as described under Materials and Methods in an attempt to establish the presence of an EP chromophore covalently bound to the cytochrome P-450 protein after incubation with EP and NADPH. After centrifugation, fractionation on Sephadex G-25, and hexane extraction, the microsomal protein that had been incubated with EP and NADPH still displayed a distinct absorption peak at 344 nm. Excitation at this wavelength produced an emission maximum at 420 nm (pH 7.5). This observation is consistent with covalent binding of an EP derivative to the microsomal protein following incubation in the presence of NADPH. On the other hand, the spectral analyses of the hexane extracts of the chromatographed protein fraction demonstrated the presence of EP and phenolic EP metabolites. Thus both EP and EP metabolites bind noncovalently to the microsomal protein and remain associated with this protein during centrifugation and gel filtration. Spectral analyses of hexane extracts of the chromatographed protein fraction from the control experiments, in which EP was mixed with microsomes in the absence of NADPH, also showed the presence of EP. After extraction with hexane, the protein-containing fraction in the control experiments did not show a distinct absorption maximum at 344 nm; excitation at 344 nm, however, produced an emission maximum at 398 nm (pH 7.5). Due to the strong noncovalent association observed between EP and the microsomal protein, the experiments reported here do not definitively establish that an EP molecule becomes covalently bound to cytochrome P-450 after incubation in the presence of NADPH.

Discussion

The results described above establish that EP can serve as substrate for an NADPH-dependent reaction catalyzed by liver microsomes (Figure 2). Some of the products of this reaction are fluorescent and in 1 N sodium hydroxide display an excitation maximum at 430 nm and a emission maximum at 498 nm. Due to the nature of the assay used (Nebert & Gelboin,

1968), the fluorescent product(s) detected should be acidic. The formation of fluorescent EP products is catalyzed by liver microsomes isolated from rats pretreated with BF or with PB, as well as from control animals.

The metabolism of the unsubstituted pyrene molecule by liver microsomal preparations from rats has been carefully characterized by Jacob et al. (1982). These workers found that the major metabolites are 1-hydroxypyrene and 4,5-dihydroxy-4,5-dihydropyrene. 1-Hydroxypyrene is the major metabolite formed by liver microsomes from control animals. When rats are pretreated with a variety of P-450-inducing agents, including BF, BP, and 3,3′,4,4′-tetrachlorobiphenyl, the formation of 1-hydroxypyrene is stimulated, and this metabolite is even more predominant. Pretreatment with PB, however, causes a 5-fold increase in oxidation at the "K region" of pyrene, and the 4,5-dihydrodiol of pyrene becomes the major metabolite. Secondary oxidation of the major 1-hydroxypyrene and 4,5-dihydroxy-4,5-dihydropyrene products yields minor diphenol and triol products.

The fluorescent products observed from EP metabolism have not been characterized; however, the formation of fluorescent phenolic products[2] from EP is consistent with the observations of Jacob et al. (1982). It can be seen from Figure 1 how reactive orientations differing from c could produce 1-ethynyl-10-hydroxypyrene or 1-ethynyl-6-hydroxypyrene. The more rapid formation of such products by liver microsomes from BF-pretreated rats is also consistent with the finding of Jacob et al. (1982) that inducers of cytochrome P-450 such as BF, BP, and 3,3′,4,4′-tetrachlorobiphenyl increase the amounts of the 1-hydroxypyrene product produced from an unsubstituted pyrene substrate.

The observations of Jacob et al. (1982), however, cannot explain the rapid loss of enzyme activities toward both EP and BP as substrates that occurs when EP is incubated with liver microsomes in the presence of an NADPH-generating system (Figure 2–5). Jacob et al. (1982) carried out 30-min incubations with 50 μM pyrene and apparently did not observe significant inhibition; these workers report that 1-hydroxypyrene does not inhibit the pyrene oxidations. We conclude that the inhibitions produced by EP are due to the presence of the ethynyl portion of this substrate. Furthermore, the fact that the inhibited activities can be restored to the original values by addition of fresh microsomal protein (Figures 2b and 3) establishes that the loss of activity is not due to lack of substrate or NADPH or to the formation of strongly inhibitory, reversibly bound, metabolites. The data in Figure 4 also show that the observed loss of BP hydroxylase activity due to EP is dependent upon NADPH and is irreversible under the incubation conditions. These observations are consistent with the view that the ethynyl group of EP is causing the self-catalyzed inactivation (suicide inhibition) of the cytochrome P-450 dependent hydroxylation activities as proposed by Ortiz de Montellano & Kunze (1980a) for a series of previously investigated alkynes.

Assays of BP hydroxylation in the presence of varying amounts of EP show that this monooxygenase reaction is more strongly inhibited in the microsomal preparations from BF-pretreated animals than from PB-pretreated animals (Figure 5; Tables I and II). Plots of the $t_{1/2}$ values calculated from the measured rates of the loss of BP hydroxylase activity vs. $[EP]^{-1}$ give straight lines as illustrated in Figure 7 and establish that the observed loss of hydroxylase activity occurs by processes that have a first-order dependence on EP. Only a single such process was detected, kinetically, in the microsomal preparations from BF-pretreated animals. Two separate first-order processes were, however, detected in the microsomal preparations from PB-pretreated animals. The losses of activity in the microsomes from PB-exposed animals occurred by slower, first-order processes (Figures 5–7; Table I). In addition to the first-order decrease in BP hydroxylase activity, determinations of the hydroxylation rates at zero time (Figure 6a,b) indicate that EP also inhibits the hydroxylase activity initially present in the microsomes.

The initial inhibition of BP hydroxylase activity by EP *and* then the irreversible loss of this activity by processes that are first-order in EP are consistent with the proposal that this alkyne functions as a substrate that can cause the suicide inhibition of this P-450-dependent monooxygenase. Furthermore, the amounts of activity lost under comparable incubation conditions (Table II) demonstrate that the effect of EP is stronger in microsomal preparations from BF-pretreated rats than in those from BP-pretreated rats. The experimental observations (Figures 5–7; Tables I and II) suggest that incubation with EP preferentially causes destruction of the major cytochrome P-450 dependent BP hydroxylase in the liver microsomes from BF-pretreated rats and of minor P-450-dependent hydroxylases in the liver microsomes from PB-pretreated rats.

The cytochrome P-450 dependent monooxygenases that are induced in rat liver by exposure to BF and that have high specificity toward BP have been purified and characterized (Saito & Strobel, 1981; Lau & Strobel, 1982; Guengerich et al., 1982). It has been concluded that the major cytochrome P-450 induced in rat liver by BF is identical with the major form, P-450$_c$, induced by 3-methylcholanthrene (Lau et al., 1982; Guengerich et al., 1982). As illustrated in Figure 1, the preferential suicide inhibition of this cytochrome P-450 form by EP can be explained in terms of the catalytic site proposed by Jerina et al. (1982) for P-450$_c$. It should be noted, however, that the observed selectivity of the inhibitory effect of EP can also be predicted by the studies of Jacob et al. (1982) on pyrene metabolism. The binding of EP to the P-450 isozyme induced by BF, which preferentially yields 1-hydroxypyrene, should position the critical 1-ethynyl group proximate to the iron-activated oxygen atom, while binding of EP to the isozyme(s) induced by PB, which preferentially catalyze pyrene oxidation at the 4,5-positions, would not be expected to place the carbon–carbon triple bond close to the activated oxygen as required for the self-catalyzed inactivation process [see Ortiz de Montellano & Kunze (1980a)].

Regardless of the exact design of the catalytic region of the cytochrome P-450$_c$ dependent monooxygenase, it is important to emphasize that the analysis illustrated in Figure 1 may be a productive approach to the design of selective P-450 suicide inhibitors [see also Nagahisa et al. (1983)]. Previously, inhibitors of this type have been reported to selectively effect the P-450 forms present in preparations from animals in which P-450 isozymes were induced by PB; Benrekassa & Decloitre (1983) found that 10 μM 17α-ethinylestradiol significantly inhibited the metabolism of BP in hepatocytes from both control rats and from PB-pretreated rats but that pretreatment with 3-methylcholanthrene rendered the BP metabolism in the isolated hepatocytes insensitive to inhibition by 100 μM 17α-ethinylestradiol. The data in Table II also show that 1 mM 4-phenyl-1-butyne inhibits the BP hydroxylase activity

[2] An alternative acidic product would be pyrenylacetic acid (Ortiz de Montellano & Kunze, 1980b). It is judged that the fluorescence maxima in sodium hydroxide (excitation, 430 nm; emission, 498 nm) and the observed shift to lower wavelengths upon acidification to pH 5.5 (excitation, 330 nm; emission, 398 nm) are more consistent with phenolic EP products.

in microsomes from BF- and from PB-pretreated rats to similar extents [see also Ortiz de Montellano & Correia (1983)].

Upon the basis of the assay results discussed above and the results previously published by Ortiz de Montellano & Kunze (1980a), by Nagahisa et al. (1983), and by Bradshaw et al. (1978), the observations summarized in Table II are unexpected. While incubation of microsomes from BF-pretreated rats with 120 μM EP for 20 min leads to the loss of 90% of the BP hydroxylation activity, under comparable incubation conditions only about 6% of the cytochrome P-450 content of these microsomes is lost. 4-Phenyl-1-butyne was used as a reference standard in these studies. We observed that incubation with 1 mM 4-phenyl-1-butyne for 20 min caused the loss of 27% of the cytochrome P-450 content of microsomes from PB-pretreated animals; Ortiz de Montellan & Kunze (1980a) reported the loss of 49% of the P-450 content in similar experiments. The results in Table II lead us to conclude that loss of P-450 content is an insensitive measure of the decrease in BP hydroxylation activity. This conclusion is consistent with the recent report of Benrekassa & Decloitre (1983), who found that the loss of cytochrome P-450 content caused by incubation with 17α-ethinylestradiol was insignificant under conditions where the P-450-dependent metabolism of BP was strongly suppressed. Furthermore, the data in Table II indicate that the observed loss of P-450 content from microsomes may show a different selectivity than that for the inhibition of a P-450-dependent enzymatic reaction. Even though EP more strongly inhibits BP hydroxylation in microsomes from BF-pretreated rats, EP causes twice as great a loss of P-450 content in the microsomes from PB-pretreated rats. The loss of P-450 content due to 4-phenyl-1-butyne in microsomes from PB-pretreated rats is also about twice that observed in microsomes from BF-pretreated rats even though the inhibition of BP hydroxylase activity in these two preparations is nearly equivalent.

The data in Table III establish that exposure of rats to EP for 96 h in vivo does not cause induction of cytochrome P-450 in liver. The data also indicate that exposure of rats to EP under the conditions used in this experiment does not eliminate P-450-dependent monooxygenase activities that are strongly inhibited by EP in in vitro assays.

Ortiz de Montellano and his co-workers have proposed that the *self-catalyzed destruction* of the heme groups of cytochrome P-450 containing enzymes by alkynes occurs by the nucleophilic attack of a heme nitrogen atom upon an electron-deficient intermediate produced by addition of the oxene oxygen to the carbon–carbon triple bond (Ortiz de Montellano & Kunze, 1980a, 1981a; Ortiz de Montellano et al., 1982). This process results in an alkylation of the heme nitrogen, which destroys the P-450 chromophore and produces new green pigments, several of which have been characterized by Ortiz de Montellano and his associates (Ortiz de Montellano & Kunze, 1980a, 1981a; Ortiz de Montellano et al., 1982). Such a mechanism for the *suicide inhibition* of cytochrome P-450 dependent enzymatic activities requires that the inhibition of the enzymatic activities and the loss of the P-450 content occur in parallel. The data in Table II indicate that this does not occur when microsomes are incubated with EP and NADPH. Nevertheless, we believe that the data in Figures 1–7 and Table I support the proposal that the observed decreases in microsomal monooxygenase activities caused by EP are the result of self-catalyzed inactivations of cytochrome P-450 dependent activities by reactive intermediates generated during enzyme-catalyzed transformations of EP. We propose that, at least with some alkyne substrates, the suicide inhibition of P-450-dependent enzymes may result from reaction of the intermediates with nucleophilic groups other than the heme nitrogen and that therefore the enzyme activity might be destroyed without a measured loss of P-450 content. This important possibility deserves further investigation. In their study of acetylenic inhibitors of cytochrome P-450$_{scc}$, Nagahisa et al. (1983) observed both suicide inhibition of cholesterol side-chain cleavage and loss of the cytochrome P-450 chromophore but concluded that alkylation of an amino acid residue at the active site was involved because no evidence for a new chromophore due to porphyrin derivatization was detected. The presence of an EP chromophore that remains associated with the microsomal protein during centrifugation, gel filtration, and hexane extraction is consistent with the formation of a covalently modified P-450 protein in the presence of EP and NADPH. Unfortunately, the extensive *noncovalent association* of EP and the microsomal protein also observed does not permit us to conclude, on the basis of these experiments, that EP becomes covalently bound to cytochrome P-450 during the NADPH-dependent metabolism.

Cytochrome P-450 inhibitors with special properties have been useful in the investigations of the P-450-dependent monooxygenases [see, for example, the review by Wiebel (1980)]. Because of the special effects of EP upon microsomal BP hydroxylase activities, this compound may prove to be a useful addition to the list of selective monoooxygenase inhibitors available to the investigators in this field. In addition, since cytochrome P-450 dependent oxidations of BP and other polycyclic aryl hydrocarbons are critical steps in the conversion of these procarcinogens into their ultimate carcinogenic metabolites [reviewed by Yang et al. (1978) and Harvey (1981)], EP or related structures may eventually be useful anticancer agents. Since it is unlikely that our initial efforts have produced the most potent or the most selective cytochrome P-450 inhibitor of this type, we believe the results described here should encourage further investigations in this important area.

Registry No. EP, 34993-56-1; BP hydroxylase, 9037-52-9; pyrene, 129-00-0; 1-formylpyrene, 3029-19-4; (bromoethyl)triphenylphosphonium bromide, 7301-93-1.

References

Benrekassa, J., & Decloitre, F. (1983) *Biochem. Pharmacol. 32*, 347–353.

Bradford, M. (1976) *Anal. Biochem. 72*, 248–254.

Bradshaw, J. J., Ziman, M. R., & Ivanetich, K. M. (1978) *Biochem. Biophys. Res. Commun. 85*, 859–866.

Ganu, V. S., & Alworth, W. L. (1978) *Biochemistry 17*, 2876–2881.

Guengerich, F. P., Dannan, G. A., Wright, S. T., Martin, M. V., & Kaminsky, L. S. (1982) *Biochemistry 21*, 6019–6030.

Harvey, R. G. (1981) *Acc. Chem. Res. 14*, 218–226.

Jacob, J., Grimmer, G., Raab, G., & Schmoldt, A. (1982) *Xenobiotica 12*, 45–53.

Jerina, D. M., Michaud, D. P., Feldmann, R. J., Armstrong, R. N., Vyas, K. P., Thakker, D. R., Yagi, H., Thomas, R. E., Ryan, D. E., & Levin, W. (1982) in *International Symposium on Microsomes and Drug Oxidations (Microsomes, Drug Oxidation, and Drug Toxicity), 5th* (Sato, R., & Kato, R., Eds.) pp 195–201, Japan Scientific Societies Press, Tokyo.

Lau, P. P., & Strobel, H. W. (1982) *J. Biol. Chem. 257*, 5257–5262.

Lau, P. P., Pickett, C. B., Lu, A. Y. H., & Strobel, H. W. (1982) *Arch. Biochem. Biophys. 218*, 472–477.

Lu, A. Y. H., & West, S. B. (1980) *Pharmacol. Rev. 31*, 277–295.

Matsumoto, M., & Kuroda, K. (1980) *Tetrahedron Lett. 21*, 4021-4024.
Nagahisa, A., Spencer, R. W., & Orme-Johnson, W. H. (1983) *J. Biol. Chem. 258*, 6721-6723.
Nebert, D. W., & Gelboin, H. V. (1968) *J. Biol. Chem. 243*, 6242-6249.
Nesnow, S., Fahl, W. E., & Jefcoate, C. R. (1977) *Anal. Biochem. 80*, 258-266.
Omura, T., & Sato, R. (1964) *J. Biol. Chem. 239*, 2370-2378.
Ortiz de Montellano, P. R., & Kunze, K. L. (1980a) *J. Biol. Chem. 255*, 5578-5585.
Ortiz de Montellano, P. R., & Kunze, K. L. (1980b) *J. Am. Chem. Soc. 102*, 7373-7375.
Ortiz de Montellano, P. R., & Kunze, K. L. (1981a) *Biochemistry 20*, 7266-7271.
Ortiz de Montellano, P. R., & Mico, B. A. (1981b) *Arch. Biochem. Biophys. 206*, 43-50.
Ortiz de Montellano, P. R., & Correia, M. A. (1983) *Annu. Rev. Pharmacol. Toxicol. 23*, 481-503.
Ortiz de Montellano, P. R., Kunze, K. L., Beilan, H. S., & Wheller, C. (1982) *Biochemistry 21*, 1331-1339.
Ryan, D., Thomas, P. E., & Levin, W. (1980) *J. Biol. Chem. 255*, 7941-7955.
Saito, T., & Strobel, H. W. (1981) *J. Biol. Chem. 256*, 984-988.
Still, W. C., Kahn, M., & Mitra, A. (1978) *J. Org. Chem. 43*, 2923-2925.
Tanikawa, K., Ishizuka, I., & Suzuki, K. (1968) *Bull. Chem. Soc. Jpn. 41*, 2719-2722.
Thomas, P. E., Korzeniowski, D., Ryan, D., & Levin, W. (1979) *Arch. Biochem. Biophys. 192*, 524-532.
Thomas, P. E., Reik, L. M., Ryan, D. E., & Levin, W. (1981) *J. Biol. Chem. 256*, 1044-1052.
van Bladern, P. J., Armstrong, R. N., Cobb, D., Thakker, D. R., Ryan, D. E., Thomas, P. E., Sharma, N. D., Boyd, D. R., Levin, W., & Jerina, D. M. (1982) *Biochem. Biophys. Res. Commun. 106*, 602-609.
Viswanathan, T., & Alworth, W. L. (1981) *J. Med. Chem. 24*, 822-830.
Vollmann, H., Becker, H., Corell, M., & Streeck, H. (1937) *Justus Liebigs Ann. Chem. 531*, 1-159.
Wiebel, F. J. (1980) *Carcinog.—Compr. Surv. 5*, 57-84.
Yang, S. K., Deutsch, J., & Gelboin, H. V. (1978) in *Polycyclic Hydrocarbons and Cancer* (Gelboin, H. V., & Tso, P. O. P., Eds.) Vol. 1, pp 205-231, Academic Press, New York.

Spectroscopic Investigation of Ligand Interaction with Hepatic Phenylalanine Hydroxylase: Evidence for a Conformational Change Associated with Activation†

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ABSTRACT: We have examined the interaction of phenylalanine hydroxylase with phenylalanine, tetrahydropterin cofactors, and an activating phospholipid, lysophosphatidylcholine. Incubation of native phenylalanine hydroxylase with phenylalanine or lysophosphatidylcholine results in an increase in the fluorescence emission of the enzyme at 360 nm, which closely parallels the increase in tetrahydrobiopterin-dependent activity observed under these conditions. The presence of tetrahydrobiopterin in the absence of phenylalanine results in quenching of the enzyme fluorescence emission; this quenching exhibits a sharp end point at about 1 mol of tetrahydrobiopterin bound/mol of enzyme subunit. The binding of tetrahydrobiopterin under these conditions is unexpectedly tight, with an estimated K_D of 10-20 nM, while in the presence of lysophosphatidylcholine, the K_D is increased to about 25 μM. Quenching experiments with sodium iodide indicate greater exposure of tryptophan residues in the phenylalanine-activated enzyme. The ultraviolet difference spectrum of phenylalanine hydroxylase in the presence of phenylalanine exhibits a peak at 238 nm, which correlates with the fluorescence increase and activation, as well as additional changes in the aromatic region, which do not correlate well with activation. Phenylalanine does not alter the far-ultraviolet circular dichroism spectrum of phenylalanine hydroxylase. In contrast, lysophosphatidylcholine appears to induce a dramatic change in enzyme secondary structure upon activation. These results suggest that activation of phenylalanine hydroxylase results in a conformation change and the exposure of buried tryptophan(s) and possibly a cysteine residue.

Phenylalanine hydroxylase, as the first and rate-limiting step in the hepatic catabolism of phenylalanine (Milstien & Kaufman, 1974), is subjected to strict metabolic control. In the presence of the natural cofactor, (6*R*)-L-*erythro*-tetrahydrobiopterin (BH_4)[1] (Kaufman, 1963), the enzyme only expresses a small fraction of the activity observed with synthetic cofactors such as 6,7-dimethyltetrahydropterin (Kaufman, 1970) and 6-methyltetrahydropterin (6-MPH_4) (Hasegawa & Kaufman, 1982). A number of treatments are known,

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[1] Abbreviations: BH_4, (6*R*)-L-*erythro*-5,6,7,8-tetrahydrobiopterin; 6MPH_4, 6-methyl-5,6,7,8-tetrahydropterin; DTT, dithiothreitol; phenylalanine hydroxylase, L-phenylalanine,tetrahydropteridine:oxygen oxidoreductase (4-hydroxylating) (EC 1.14.16.1); Pipes, 1,4-piperazinediethanesulfonic acid; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride.