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DIPHENYLOXAZOLE METABOLISM BY ARYL HYDROCARBON HYDROXYLASE

THESIS

Presented to the Graduate Council of the North Texas State University in Partial Fulfillment of the Requirements

For the Degree of

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By

Mary E. Abreu, B. A.

Denton, Texas

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2,5-Diphenyloxazole (PPO) was tested as a potential alternate inducer for the aryl hydrocarbon hydroxylase (AHH) system. Its apparent lack of carcinogenicity and toxicity provide a possible system for investigation of enzyme systems related to chemical carcinogenesis without exposure of the researcher to potent carcinogenic compounds. These studies found PPO to be an inducer of AHH in cultured human lymphocytes. When PPO was utilized as a substrate for the AHH assay system, the major metabolites produced were strongly fluorescent. A simple fluorometric assay was developed which employed PPO as the substrate and which measured constitutive activity more efficiently than similar assays using benzo(α)pyrene as the substrate. Quantitation of both basal and induced lymphocyte AHH metabolism of PPO may be applicable to human population studies and may provide a tool to determine possible genetic variables with respect to carcinogen metabolism related to cancer risk.

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CHAPTER I

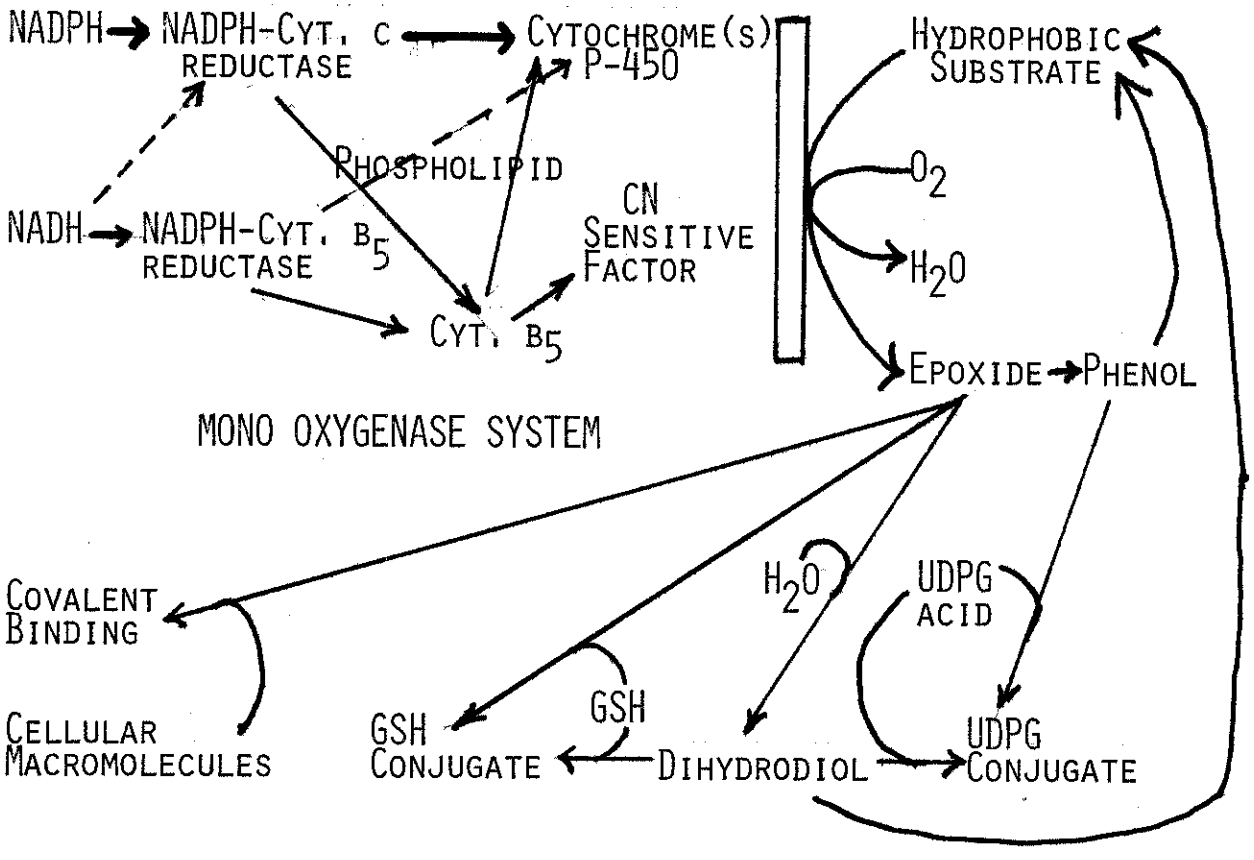
INTRODUCTION

Chemical carcinogenesis was first documented in man in 1775 by the British physician Percival Potts, who attributed the high incidence of scrotal cancer in London chimney sweeps to their contact with soot and coal tar (71). Since that time, at least 1,000 chemicals have been shown to induce cancer in a wide variety of animals and to cause transformation in cells of cultured tissues (16, 25). As our civilization has become more industrialized, the environment has become increasingly contaminated with a number of cancer-producing chemicals. Epidemiologists estimate that approximately 70-90% of human cancer is caused by environmental pollutants such as the polycyclic aromatic hydrocarbons (PAH) and related compounds (56).

Most PAH are not in themselves carcinogenic until after they are metabolically converted to reactive electrophilic forms which are capable of covalent binding with proteins and nucleic acids, a step which apparently preceeds tumor initiation (9, 24, 31, 70). PAH, which are the products of incomplete combustion, are found in particulate air pollutants (22), in smoked foods (47),

in water (4), and in condensates of smoke, including cigarette smoke (84).

PAH, as well as a wide variety of other xenobiotics, are metabolized by a mixed-function oxygenase system known as aryl hydrocarbon hydroxylase, (AHH), or benzo(α)-pyrene hydroxylase, which is present in most mammalian tissues (16, 60). A scheme for the function of this multi-enzyme complex with possible pathways for hydrophobic substrates has been suggested by Nebert et al (62) and is illustrated in the following figure.



The AHH complex apparently functions in a tightly structured steric configuration on the membranes of the endoplasmic reticulum and requires NADPH and molecular oxygen for enzymatic activity (21, 55). AHH has been solubilized from mammalian liver microsomal preparations and resolved into three major fractions, cytochrome P-450, NADPH cytochrome c reductase, and phospholipid (51, 52, 78). The substrate specificity of this enzyme system resides in the cytochrome P-450 fraction (29, 36). Multiple forms of the hemeprotein (cytochrome P-450) exist in liver microsomes obtained from the same animal, as documented by purification procedures (15, 17) and by spectral studies (28, 30).

An important characteristic of AHH is its inducibility. The level of enzyme fluctuates depending on an organism's exposure to specific inducing agents, which include exogenous (e.g. PAH, drugs, pesticides) as well as endogenous (e.g. steroid hormones) chemicals (16, 23). The exact mechanism whereby AHH inducers increase enzyme activity is still not known, however, it has been reported that AHH induction follows the synthesis of mRNA followed by de novo protein synthesis (36, 61, 81), which is associated with increases in the cytochrome P-450 fraction, and which results from exposure to "inducers" (1,7).

P-450 inducers have conventionally been classified as type I or type II, depending on the predominant species

of cytochrome P-450 induced (15). Pre-treatment of experimental animals with a type I inducer such as phenobarbital results in a general increase in the metabolism of PAH, as well as numerous other drugs, by the hepatic AHH system (16). Studies utilizing AHH inhibitors (83) and those which determined metabolite profiles (72, 73) indicated that the type I induced hepatic enzyme may be the same as the constitutive enzyme (26). Hepatic tissue appears to be the only tissue in which AHH activity is increased by type I compounds (60).

Type II inducers include the PAH. Pre-treatment of experimental animals with PAH results in a specific increase in PAH metabolism by hepatic and extrahepatic tissues. Of these, the greatest enzyme increase is in the hepatic tissues (60, 66). Metabolism of most other substrates is unchanged or is slightly decreased during PAH induction (52). The changes in enzyme activity reflect changes in both the V_{max} and K_m for various substrates (35). Type II induction has been characterized by an increase in the cytochrome moiety with a shift of the absorbance maximum of the reduced, CO-bound, cytochrome from 450 to 448 nm (1, 65). This modified hemeprotein also exhibits differences in electron paramagnetic resonance (EPR) characteristics (67) and in the response to different enzyme inhibitors (77, 82). The type or types of cytochrome P-450 induced by xenobiotics determine the

distribution of metabolites (41, 49, 74).

Benzo(α)pyrene (BP) is a prototype PAH which has been extensively studied. The strong fluorescence of its phenolic metabolites forms the basis for a sensitive assay of AHH (57). BP is metabolized to epoxides (33), phenols, and quinones by AHH (26). These products may be further metabolized to dihydrodiols or water-soluble metabolites by hydratases or transferases (57, 64). The initial reaction catalyzed by AHH results in the formation of at least three different epoxide intermediates at the 4-5, 7-8, and 9-10 positions of BP (73). Newly formed epoxides may interact with DNA (24), undergo nonenzymatic conversion to phenols, or be further metabolized to non-toxic dihydrodiols (18, 41). Water-soluble metabolites are formed when there is enzymatic conversion of oxygenated intermediates by glutathione S-epoxide transferase (34, 63). The extent of covalent binding of the epoxides of BP to DNA has been correlated with their ability to produce malignant transformation (32, 48, 53). In the case of BP the 7,8-dihydrodiol, 9,10-epoxides of BP are currently considered to be the ultimate carcinogenic species (8, 39).

The actual mechanisms of chemical carcinogenesis cannot be determined <u>in vivo</u>, but a number of cell culture systems have been established to facilitate these studies.

Chemical carcinogenesis in vitro was first reported by Berwald and Sachs (6) who demonstrated the transformation of hamster embryo cells with PAH. Another system was independently developed by Chen and Heidelberger, (14), using PAH transformable C3H mouse ventral prostate cells. Chinese hamster cells (37, 45) and bacteria (3) have also been utilized to study mutagenic activities of PAH. Initial findings from these studies suggest a strong correlation between the mutagenic activities of the dihydrodiol-epoxides of PAH and their capacity to cause cellular transformation (32, 53, 76).

A great deal of effort is currently directed toward establishing tests in humans which could be used to compare AHH activity with lung cancer susceptibility. Two major problems are apparent when studying AHH activity in man: the availability of tissue and the availability of suitable assay procedures. Human tissues that have been tested include cultured embryo cells (38, 44), placenta (59, 68), foreskin (50), biopsied liver (2, 46), skin (20), pulmonary alveolar macrophages (12), blood monocytes (5), and peripheral blood lymphocytes (10, 80). Of all these tissues, human monocytes and/or mitogen-stimulated cultured lymphocytes present the most readily available tissues for assessing AHH in humans.

The current assays for measuring AHH activity include 1) measurement of conversion of BP to 3-OH BP spectrophotofluorometrically (57), 2) measurement of conversion of ^{3}H-BP to water-soluble forms (19), 3) quantitative measurement of the conversion of BP to its various metabolites using thin layer or high pressure liquid chromatography (72, 74, 75), 4) cytochemical analysis of individual cells by microfluorometry (11, 43), and 5) direct quantitation of the P-450 or P-448 cytochromes (2, 69).

Unfortunately, the current assays to determine AHH levels in cultured human lymphocytes and/or monocytes are not reproducible, a factor which is probably due to the different response of cultured lymphocytes to mitogens, the variability of mitogen preparations, the variability in fetal calf serum used in the culture medium and the variability in toxicity of the test chemicals to cultured cells.

Another feature of the AHH assays established thus far is the potential danger to researchers from accidental carcinogen exposure. There exists the need for a test for comparing cancer risk with enzyme activity which can be performed on readily available human tissue, is reproducible, safe, fast, and adaptable to clinical use. This study pursued the ultimate goal of developing such an assay.

CHAPTER II

MATERIALS AND METHODS

Experimental Animals

C57Bl/6J mice were obtained from Jackson Laboratories, Bar Harbor, Maine. Sprague-Dawley male rats were obtained from Holtzman Laboratories, Madison, Wisconsin. These animals were maintained on Purina Rat Chow and water ad libitum in the vivarium of the Biology Department of North Texas State University.

Lymphocyte Isolation and Culture

Whole blood was obtained from human volunteers by venipuncture and was diluted with one-half volume of sterile heparinized saline solution (0.9% sodium chloride solution with 10 units/ml heparin). Sterile conditions were maintained throughout the cell culture. A 15-20 ml aliquot of the blood-saline mixture was layered onto 8 ml of a solution containing 6% Ficoll and 10% sodium diatrizoate (Hypaque, Winthrop Laboratories), and centrifuged at 800 g for 10 min. The mononuclear leukocytes (lymphocytes and monocytes) were recovered from a buffer layer at the interface between the plasma and the Ficoll-Hypaque. The lymphocyte/monocyte preparation was mixed

with heparinized saline and the cells were sedimented at 300 g for 15 min. The cell pellet was resuspended in Joklik's Modified Minimum Essential Medium (Gibco F-13), containing 15% fetal calf serum (Gibco), 1% pokeweed (Gibco), 1% phytohemagglutinin (Gibco), and 50 units/ml heparin (Sigma). The cell concentration was 0.4 - 0.6 x 10^6 cells/ml and the culture medium depth was 0.4 - 0.6 cm in glass scintillation vials or 250-ml Falcon plastic culture flasks. Cell suspensions were incubated at 37°C in a humidified, 5% CO_2 atmosphere for 96 hours. Twenty-four hours prior to harvest, cell cultures received the inducing agents 1,2-benzanthracene (BA), 3-methylcholanthrene (MC), or 2,5-diphenyloxazole (PPO) at varying concentrations. These compounds were dissolved in acetone and added to the culture medium in a volume approximately 0.2% of the culture medium. The same volume of acetone was added to control cultures.

Cell Harvest and Assay

The AHH assay was that of Cantrell *et al* (13), or a modification of the method of Busbee *et al* (10). For BP and PPO metabolism, 96-hour cultures were transferred to conical centrifuge tubes and cells were sedimented at 100 g for three minutes. The cells were resuspended in fresh culture medium at pH 8.0. A 50 µl aliquot of the resuspended cells was removed for cell counts. Viability

was estimated after diluting with 50 µl of 0.4% trypan blue. Exclusion of the dye after five minutes was the criterion for viability. Two-ml aliquots of the cell suspension received either 25 µg BP or 10 µg PPO (dissolved in 10 µl acetone) and were incubated at 37°C for one hour. The reaction was stopped by addition of 8 ml of 10% acetone in hexane while vortex-mixing. A 4-ml aliquot of the organic phase was transferred to 1 ml of 1N NaOH and vortex-mixed. Fluorescence of the lower, aqueous, phase was measured in an Aminco-Bowman spectrophotofluorometer. The wavelength maxima for excitation and emission of hydroxybenzo(α)pyrene (BP-OH) were 396 nm and 522 nm, respectively. The maxima for hydroxydiphenyloxazole (PPO-OH) were 345 nm and 510 nm.

For the direct assay of hydroxybenzo(α)pyrene (13), the cells were resuspended at 10^6/ml in EMSAD buffer at pH 8.0 {25 mM 4-(2-hydroxyethyl, 1-1-piperazine propane sulfonic acid), 3 mM $MgCl_2$, 125 mM sodium chloride, 0.1% bovine serum albumin, and 10 mM dextrose}. One-ml aliquots received 2 µg BP in a volume of 10 µl of methanol and were incubated at 37°C for one hour. The reaction was stopped by adding 0.5 ml of 20% neutral formalin with gentle shaking. After allowing the cells to fix for five minutes, 0.5 ml of 1N NaOH were added and mixed vigorously. The fluorescence of the suspension was measured with excitation at 460 nm and emission at 522 nm.

AHH Induction *In Vivo*

C57Bl/6J mice or Sprague-Dawley rats were injected intraperitoneally with phenobarbital (80 mg/kg) in saline, or with 1,2-benzanthracene (50 mg/kg), 3-methylcholanthrene (20 mg/kg), or 2,5-diphenyloxazole (20, 50, or 200 mg/kg) in corn oil. Control animals received corn oil only. The animals were injected on four consecutive days prior to sacrifice.

BP and PPO Metabolism in Mouse Liver Microsomes

Experimental animals were sacrificed by cervical dislocation, and their livers were removed. Livers were diced, washed, and homogenized in cold TMS buffer, pH 7.5 (50 mM Tris-HCl, 200 mM sucrose, and 3 mM $MgCl_2$). The homogenate was centrifuged at 10,000 g for 15 minutes which removed mitochondria, nuclei, and other cellular debris. The supernatant was centrifuged at 105,000 g for 60 minutes in a Beckman Model L Preparative Ultracentrifuge equipped with a 50 Ti rotor. The microsomal pellet (disrupted endoplasmic reticulum) was resuspended in cold TMS buffer to a protein concentration of approximately 0.5 mg/ml. Dilutions were made from these preparations for metabolic studies.

Fluram (Roche Diagnostics) was used to determine

protein content of microsome preparations (79).

Assay of BP and PPO Metabolism

Duplicate 1.0-ml aliquots of microsomal suspensions were incubated with shaking at 37°C with BP or PPO in the presence of 1.0 mg NADPH under conditions indicated for the various experiments. The enzyme reaction was stopped by the addition of 3.0 ml of 10% acetone in hexane, and the tubes were vortex-mixed for 20 sec. After centrifuging to separate the phases, the upper, organic, phase was transferred to 1.0 ml of 1N NaOH in a 13 x 100 mm culture tube, vortex-mixed for 15 sec, and centrifuged to separate the phases. Fluorescence of the lower, aqueous, phase was determined in an Aminco-Bowman spectrophotofluorometer. The wavelength maxima for excitation and emission of hydroxybenzo(α)pyrene (BP-OH) were 396 nm and 522 nm, respectively. The maxima for hydroxydiphenyloxazole (PPO-OH) were 345 nm and 510 nm.

CHAPTER III

RESULTS

Excitation and emission spectra were determined for the metabolite(s) of 2,5-diphenyloxazole (PPO). The peak of excitation in 1N NaOH was observed at 345 nm with the peak of emission at 510 nm (Fig. 1a). A shoulder was seen on the emission peak at approximately 420 nm. After neutralizing the NaOH-metabolite mixture with an equal volume of 1N HCl, fluorescence peaks were shifted left to 330 nm for excitation and 400 nm for emission (Fig. 1b). In hexane, the peak of excitation was further shifted left to 312 nm, and double emission peaks appeared at 355 nm and 365 nm.

The excitation and emission spectra of PPO metabolites, which were separated by thin-layer chromatography and subsequently extracted into 1N NaOH, are shown in Figure 2. Both of these metabolites (R_f of 0.30 - 0.35) contribute to the fluorescent spectrum of the alkali-soluble fraction (Fig. 1a).

The relationship between the kinetics of metabolism and substrate concentration in hepatic microsomes from control and BA-treated mice is seen in Figures 3 and 4. The enzyme activity is expressed as fluorescence units/μg

protein (F units/μg protein), with a fluorescence unit referring to the scale reading from the spectrophotofluorometer obtained with the appropriate wavelength settings for hydroxylated BP (excitation of 396 nm and emission of 522 nm) or hydroxylated PPO (excitation of 345 nm and emission of 510 nm). AHH specific activity is conventionally expressed as ρmoles of 3-OH BP formed per minute per mg protein or per 10^6 cells. Presentation of the data as fluorescence units as a measure of substrate hydroxylation allows comparison of BP and PPO metabolism on the same relative scale.

The rate of metabolism of BP and PPO by PAH-induced hepatic microsomes was higher than that obtained with microsomes from control animals, regardless of which substrate was used (Fig. 3 and 4). At extremely low substrate concentrations (less than 1.0 μg) the apparent rate of BP or PPO metabolism by induced mouse hepatic microsomes was only slightly greater than that obtained with microsomes from control mice. A BP or PPO concentration of 10 - 25 μg was found to be saturating for both the induced and non-induced enzyme. The affinities of PPO and BP for the enzyme are within the same order of magnitude.

There was a direct relationship between the amount of substrate metabolized and the microsomal protein concentration (Fig. 5 and 6). The formation of BP

or PPO metabolites was approximately proportional to enzyme concentration over two orders of magnitude, however a strictly linear relationship can not be concluded.

Time course studies of BP and PPO metabolism by hepatic microsomes from control and BA-induced mice indicated the enzyme reaction to be approximately linear with time up to 15 minutes with either BP or PPO as substrates (Figures 7 and 8).

There was a positive relationship between kinetics of metabolite formation and substrate concentration as presented for BA-induced lymphocytes from two different blood donors (Fig. 9). BP and PPO metabolism is expressed as fluorescence (F) units/10^6 cells. PPO substrate concentrations greater than 100 μM resulted in a decreased amount of metabolite formation. BP metabolite formation did not plateau at substrate concentrations up to 200 μM.

Induction ratios obtained with varying BA concentrations using BP or PPO as substrates were calculated by dividing the values for the amount of metabolite formation in the BA-treated cells by the amount of metabolite formation in the non-induced cells (Fig. 10). The induction ratio represents the ratio of PAH-induced enzyme levels to constitutive levels. Induction ratios obtained with PPO as substrate were lower than those found when BP was employed as the substrate. In cells treated with

0.5 μM BA or less, the induction ratios were essentially the same. The largest difference between ratios of the two substrates was found at the optimal inducing concentration of BA (10 μM).

The effects of mixed-function oxidase (MFO) enzyme inhibitors on metabolism of BP and PPO were determined for both cultured human lymphocytes and rat hepatic microsomes (Tables I and II). The values in parentheses represent inhibited AHH activity expressed as a percent of control activity.

In contrast, 7,8-benzoflavone (ANF) inhibited the metabolism of BP and PPO in all three groups of lymphocytes to approximately the same extent (Table I). Inhibition was the greatest in the BA-induced lymphocytes with 96% depression of PPO metabolism and 78% depression of BP metabolism. SKF-525A depressed PPO metabolism in all three groups of lymphocytes with the most pronounced effect (decrease of 44% of PPO metabolism) being in the PB-treated cells. BP metabolism was enhanced in the presence of SKF-525A. This indicates the apparent absence of hepatic type cytochrome P-450 in cultured human lymphocytes induced with BA.

ANF depressed PPO metabolism in non-induced, PB-, and MC-treated rat liver microsomes (Table II). A 77% decrease of PPO metabolism was noted in the MC-treated

microsomes, with only a 59% decrease in BP metabolism in the non-induced microsomes, and slightly enhanced BP metabolism in the non-induced microsomes, and slightly enhanced BP metabolism in the PB-treated microsomes (17% increase). SKF-525A depressed metabolism of both substrates, with the greatest inhibition in the non-induced microsomes. BP metabolism was not inhibited by SKF-525A in MC-treated microsomes.

The effect on BP hydroxylase activity of exposure of the lymphocytes to various levels of PPO during the last 24 hours of the culture period demonstrates that the maximal extent of induction was reached with about 30 μM PPO (Fig. 11). A concentration of 100 μM gave no further induction of BP metabolism. Toxicity was not noted under these conditions. Exposure of lymphocytes to 30 μM PPO for the last 24 hours of culture resulted in a BP specific activity of 2.2 ρmoles/10^6 cells/minute.

The effect on AHH activity of changes in the concentration of BA in the culture medium in lymphocytes was toxicity at the highest BA level (Fig. 12). The optimal concentration for inducing AHH was 10 μM when BA was added to the cultures 24 hours prior to harvest. Toxicity was observed at a BA concentration of 50 μM. The range of BA concentrations required to elicit maximal enzyme induction without cytotoxicity was narrower

than that seen using PPO (Fig. 11). Exposure of the lymphocytes to 10 μM BA for 24 hours resulted in BP specific activity of 2.8 ρmoles/10^6 cells/minute. This value is 27% greater than the maximal enzyme activity induced by PPO.

A dose effect curve of MC on AHH activity in lymphocytes showed maximal induction of BP hydroxylase activity with 1 μM MC (Fig. 13). Toxicity was noticeable at MC concentrations above 2 μM.

Data shown in Figures 11, 12, and 13 represent activity in lymphocytes from different blood donors and therefore specific activity resulting from the three different inducers may not be comparable.

PPO and PAH were examined for their activities as inducers of AHH with subsequent metabolism of BP and PPO by the induced rat liver microsomes. MC pre-treatment of experimental animals resulted in a 173% increase of BP metabolism and a 32% increase of PPO metabolism (Fig. 14). PPO pre-treatment of the animals produced only a 14% increase in BP metabolism and depressed PPO metabolism by 19%. PB was intermediate between MC and PPO in its ability to induce AHH activity as indicated by a 63% increase of BP metabolism and a 24% increase of PPO metabolism.

When this treatment was repeated with immature

rats (1-2 months) and two different doses of PPO, there was a 747% increase in hydroxylation of BP and a 707% increase in metabolism of PPO (Fig. 15). PB pre-treatment increased BP metabolism by 104% and PPO metabolism by 93%. The AHH activity in hepatic microsomes from the untreated immature rats was considerably decreased when compared to control activity measured in adult rats. A PPO dose of 20 mg/kg of body weight increased BP metabolism 61% and increased PPO metabolism 31%, while a PPO dose of 200 mg/kg depressed BP metabolism by 36% and depressed PPO metabolism 2%.

A comparison of increased metabolism of PPO and BP by hepatic microsomes induced with PPO or with other AHH inducers was made using microsomes from C57Bl/6J mice (Fig. 16). The procedure was the same as that for the experiments shown in Figures 14 and 15. The strain of mice utilized is genetically responsive to treatment by AHH inducers. BP metabolism was increased 167% in the BA-induced group and 127% in the PB-induced mice. The reverse was observed when PPO was used as substrate. PPO metabolism was increased 68% in the PB-treated mice and only 37% in BA-treated animals. Both PPO and BP metabolism were decreased by approximately the same extent in the two PPO-treated groups.

Figure 1. Excitation and emission spectra of the major PPO metabolites. (a) An aliquot of the metabolite was extracted into 1.0 ml of 1N NaOH and the excitation spectrum recorded with the emission wavelength set at the emission peak. The emission spectrum was recorded with the excitation wavelength set at the excitation peak. (b) An equal volume of 1N HCl was added to the above cuvette and the excitation and emission spectra determined as indicated by the shaded peaks. Subsequent extraction into hexane produced spectra indicated by the unshaded areas.

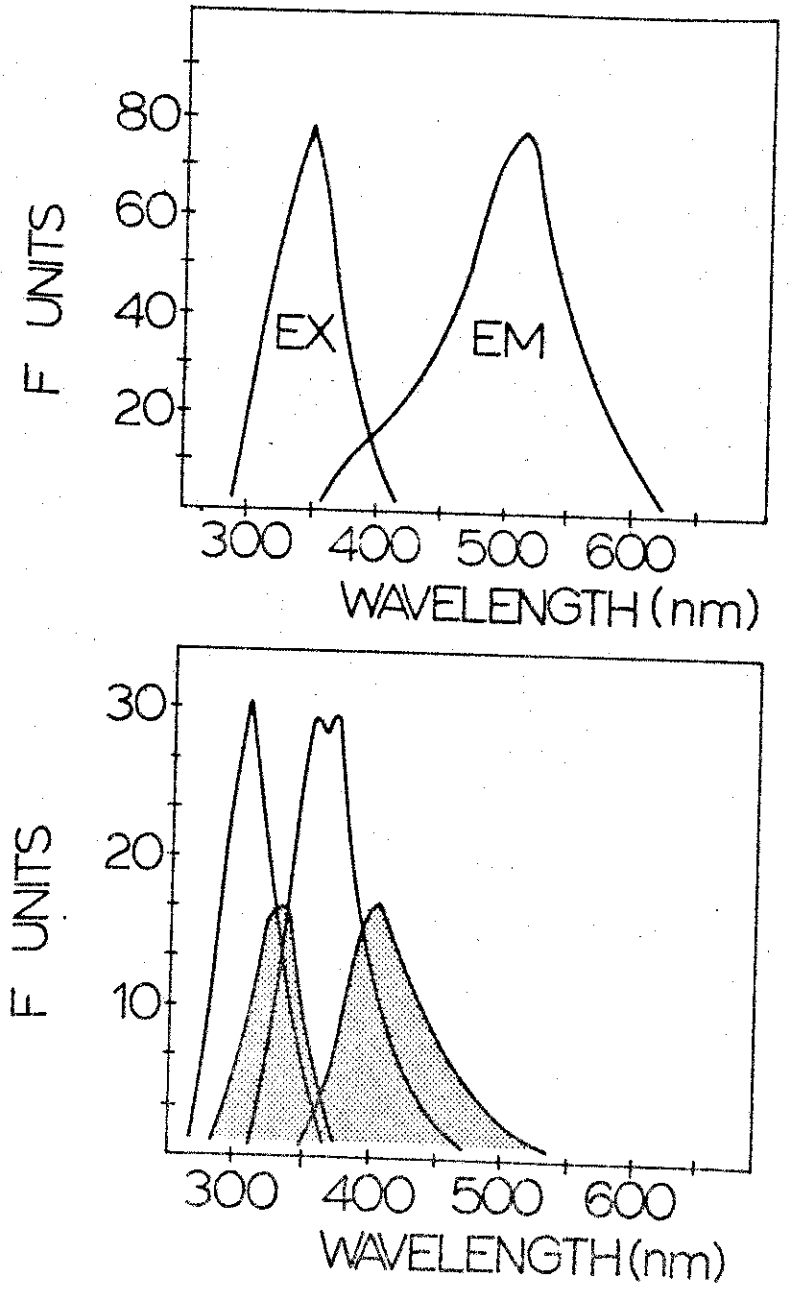

F UNITS
80
60
40
20
EX
EM
300
400
500
600
WAVELENGTH (nm)
F UNITS
30
20
10
300
400
500
600
WAVELENGTH (nm)

Figure 2. Excitation and emission spectra of separated PPO metabolites. ^{3}H-PPO (9.18 mCi/mg) and NADPH was incubated with rat hepatic microsomal preparations. PPO metabolites were extracted from the reaction mixture with ethyl acetate. After evaporating the ethyl acetate, the PPO metabolites were redissolved in acetone and spotted onto a thin-layer chromatogram (TLC). The TLC was developed in a solvent system of 8% ethanol in benzene. Fluorescent areas were marked and separately scraped from the TLC. The separated metabolites were eluted from the silica gel with hexane and extracted with 1N NaOH. The excitation and emission spectra was recorded as in Figure 1.

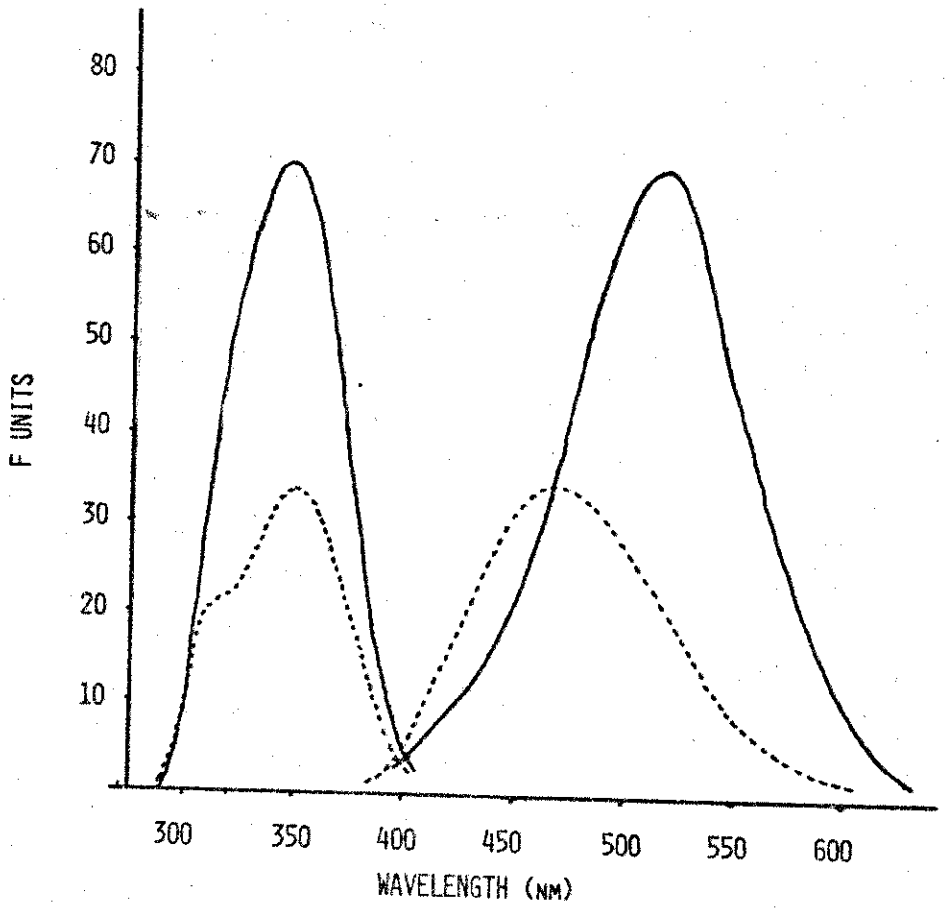

80
70
60
50
40
30
20
10
F UNITS
300
350
400
450
500
550
600
WAVELENGTH (NM)

Figure 3 BP substrate concentration curve in liver microsomes from control and BA-treated mice. Three C57Bl/6J mice were injected with BA (20 mg/kg) on three consecutive days prior to sacrifice. Liver microsome preparations containing approximately 100 µg/ml protein were also prepared from non-induced mice. These microsomal preparations were used in experiments illustrated in Figures 3-8. Duplicate 1.0 ml aliquots were incubated for 15 minutes at 37°C with 1.0 mg NADPH and varying concentrations of BP (0.1 - 100 µg). The reaction was stopped with 3 ml of 10% acetone in hexane. After vortex-mixing 30 sec and centrifuging to separate the phases, the organic phase was removed and added to 1.0 ml of 1N NaOH, mixed vigorously, and centrifuged to separate the phases. Fluorescence of the lower, aqueous phase was measured with excitation at 396 nm and emission at 522 nm.

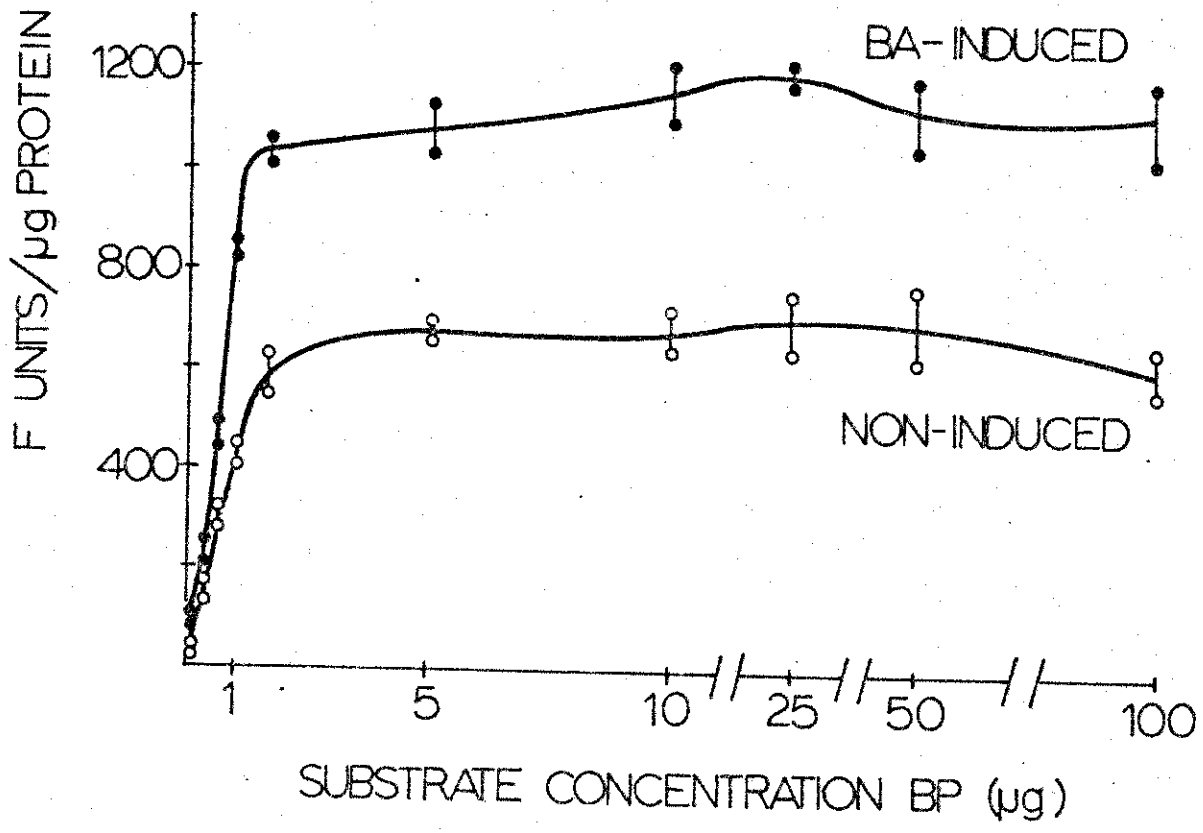

F UNITS/µg PROTEIN
1200
800
400
BA-INDUCED
NON-INDUCED
1
5
10
25
50
100
SUBSTRATE CONCENTRATION BP (µg)

Figure 4. PPO substrate concentration curve in liver microsomes from control and BA-treated mice. Duplicate 1.0 ml aliquots of induced and non-induced liver microsomes (described in Fig. 3) were incubated for 15 minutes at 37°C with 1.0 mg NADPH and varying concentrations of PPO (0.1 - 100 µg). The reaction was stopped as described in Figure 3 and fluorescence was measured with excitation at 345 nm and emission at 510 nm.

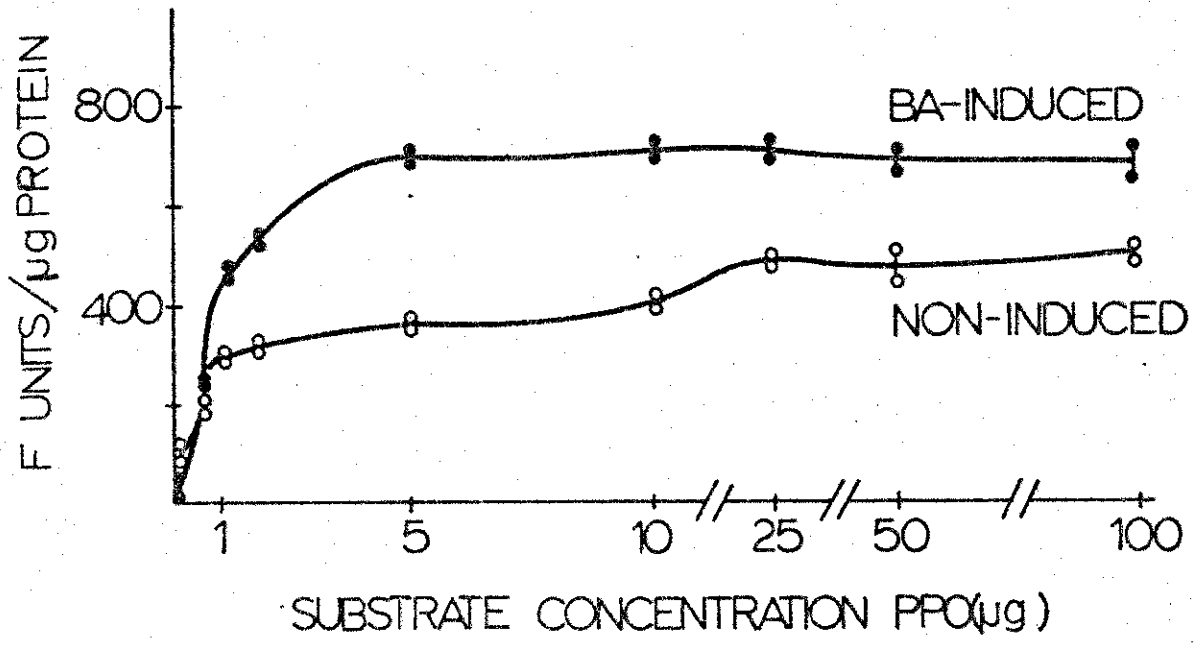

F UNITS/µg PROTEIN
800
400
BA-INDUCED
NON-INDUCED
1
5
10
25
50
100
SUBSTRATE CONCENTRATION PPO(µg)

Figure 5. Protein concentration curves for BP metabolism by BA-induced and non-induced liver microsomes. Duplicate 1.0 ml aliquots of microsomes described in Figure 3 at protein concentrations from 0.05 - 5 mg/ml were incubated 15 minutes at 37°C with 1 mg/ml NADPH and 25 µg BP. The reaction was stopped as described previously and fluorescnece determined with excitation at 396 nm and emission at 522 nm.

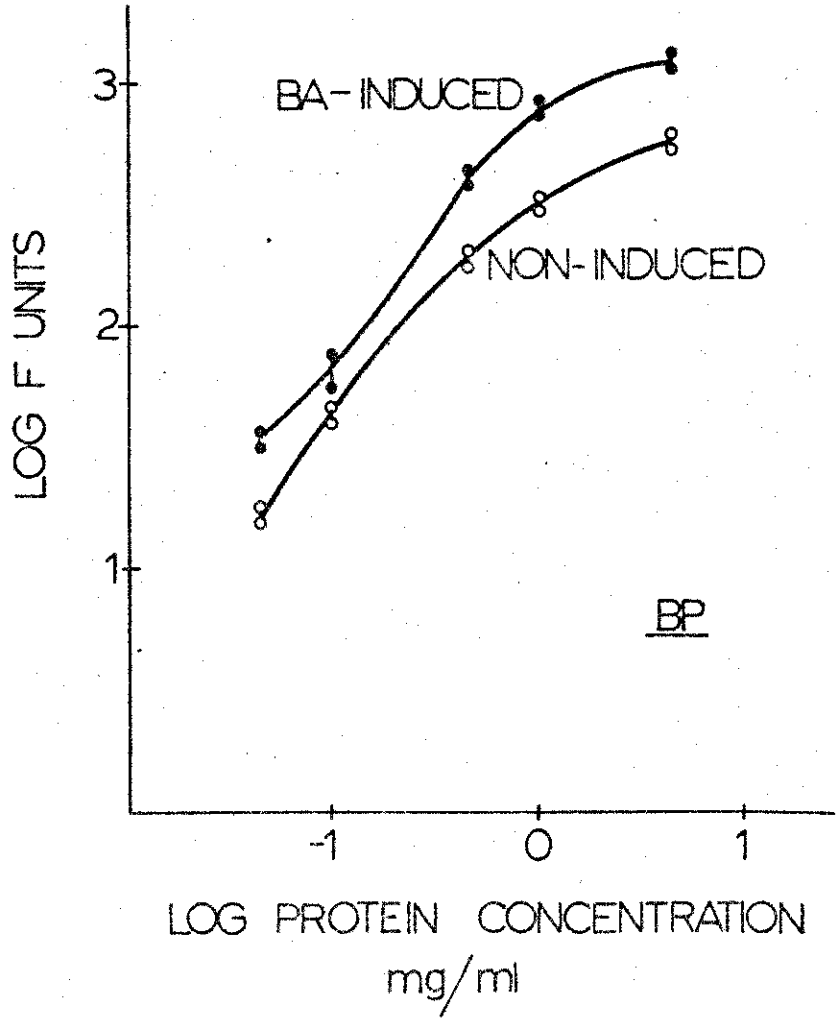

BA-INDUCED
NON-INDUCED
BP
LOG F UNITS
3
2
1
-1
0
1
LOG PROTEIN CONCENTRATION
mg/ml

Figure 6. Protein concentration curves for PPO metabolism by BA-induced and non-induced liver microsomes. The procedure was the same as indicated in Figure 5, with PPO (10 µg) substituted for BP. Fluorescence was determined with excitation at 345 nm and emission at 510 nm.

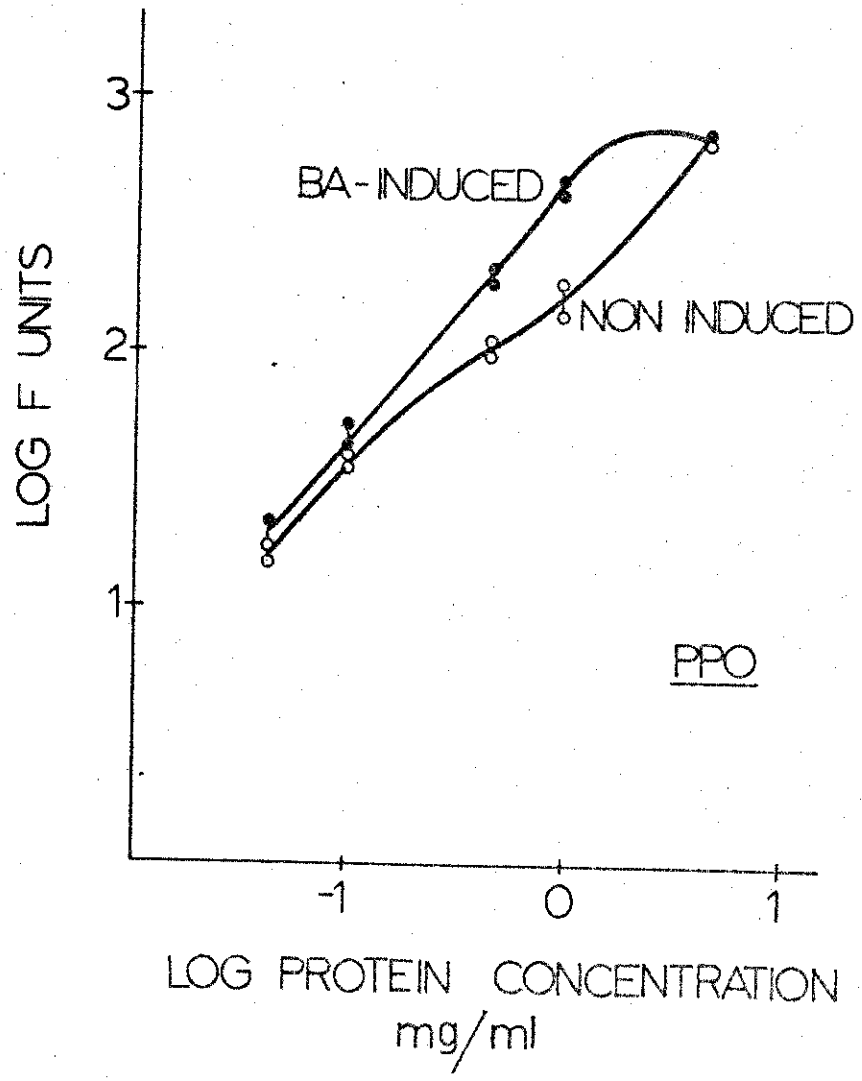

3
2
1
LOG F UNITS
BA-INDUCED
NON INDUCED
PPO
-1
0
1
LOG PROTEIN CONCENTRATION
mg/ml

Figure 7. Time course of activity in induced and non-induced microsomes with BP as substrate. Microsome preparations described in Figure 3 were utilized. Duplicate 1.0 ml aliquots were incubated various times (0, 2, 5, 10, 15, 30, or 60 minutes) with 1.0 mg NADPH and 25 µg BP. Fluorescence was determined with excitation at 396 nm and emission at 510 nm.

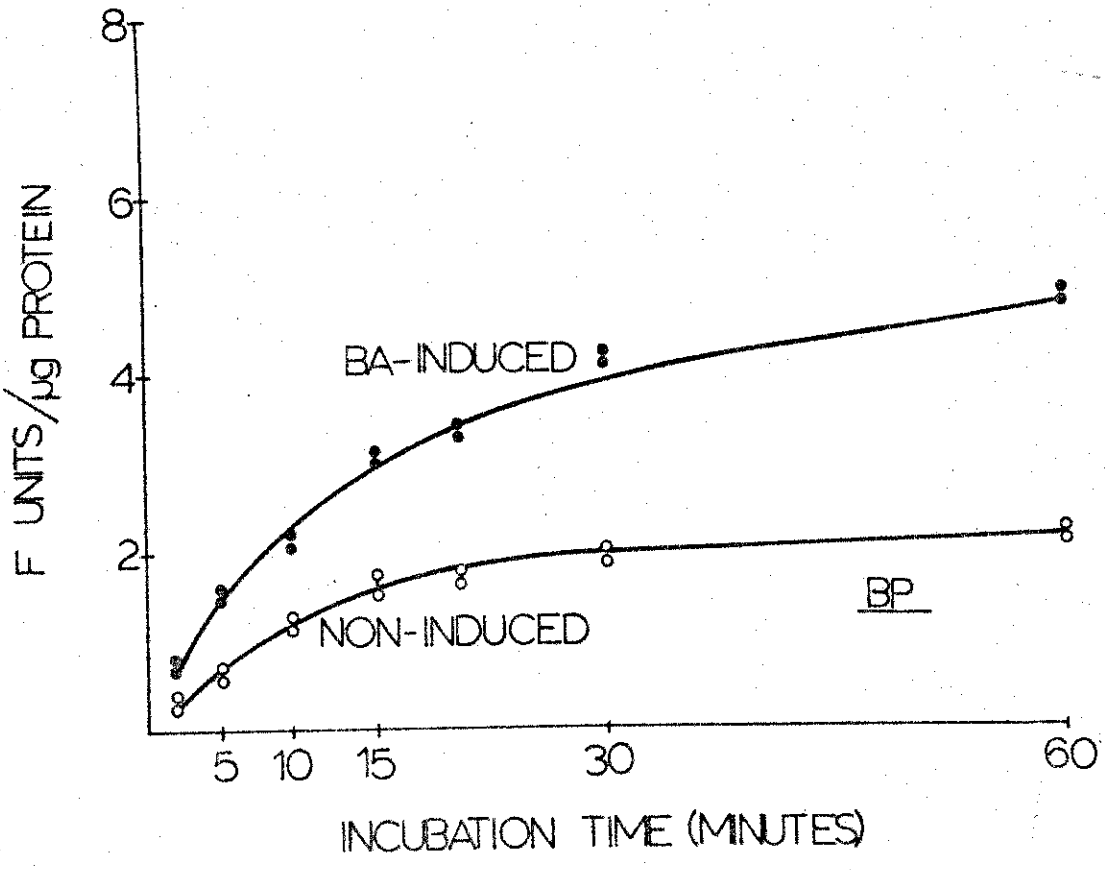

F UNITS/µg PROTEIN
8
6
4
2
BA-INDUCED
NON-INDUCED
BP
5
10
15
30
60
INCUBATION TIME (MINUTES)

Figure 8. Time course of activity in induced and non-induced microsomes with PPO as substrate. The same procedure as in Figure 7 was used, substituting PPO (10 µg) for BP. Fluorescence was determined with excitation at 345 nm and emission at 510 nm.

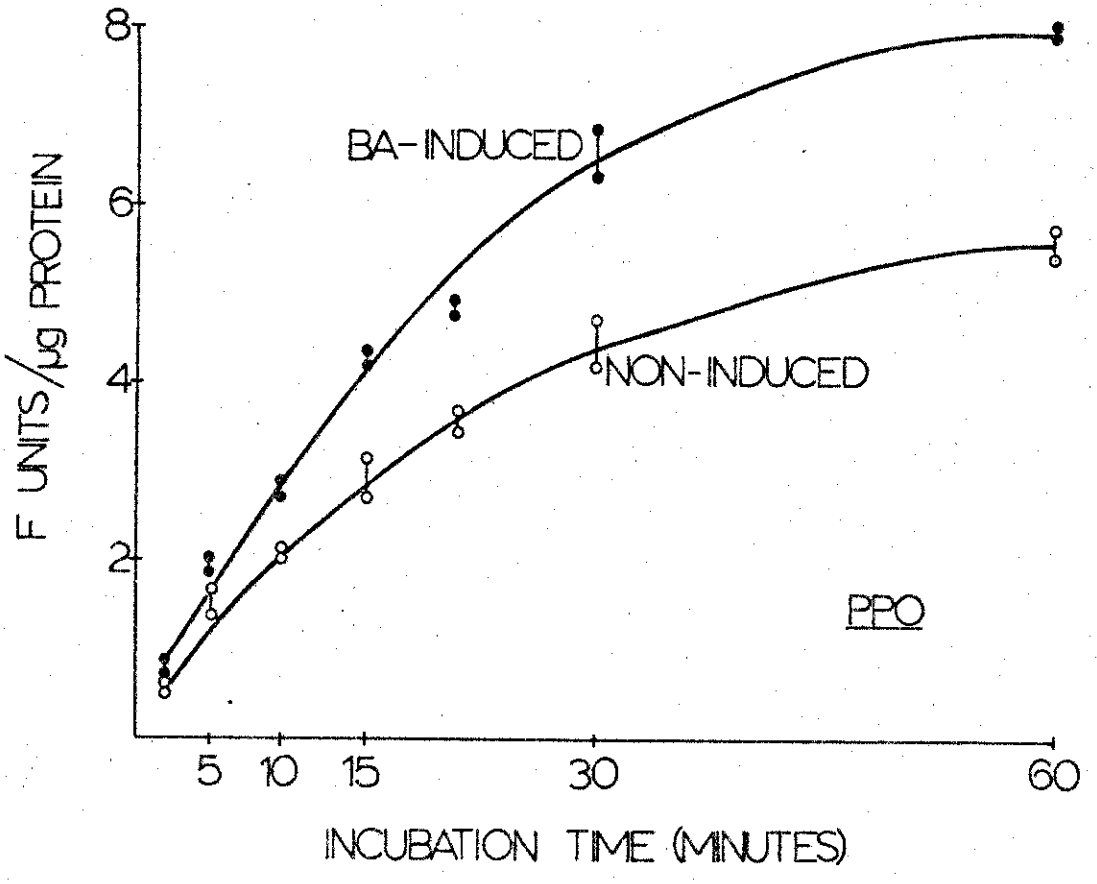

8
6
4
2
F UNITS/µg PROTEIN
BA-INDUCED
NON-INDUCED
PPO
5
10
15
30
60
INCUBATION TIME (MINUTES)

Figure 9. Substrate concentration curves of BP and PPO in BA-induced cultured human lymphocytes. Lymphocytes obtained from two different blood donors were cultured 96 hours in the presence of 10 μM BA. Cells were resuspended in EMSAD buffer (pH 8.0) at a cell concentration of 10^6/ml. Duplicate 1.0 ml aliquots were incubated at 37°C for 60 minutes with varying concentrations of BP or PPO. Fluorescent measurements were as before.

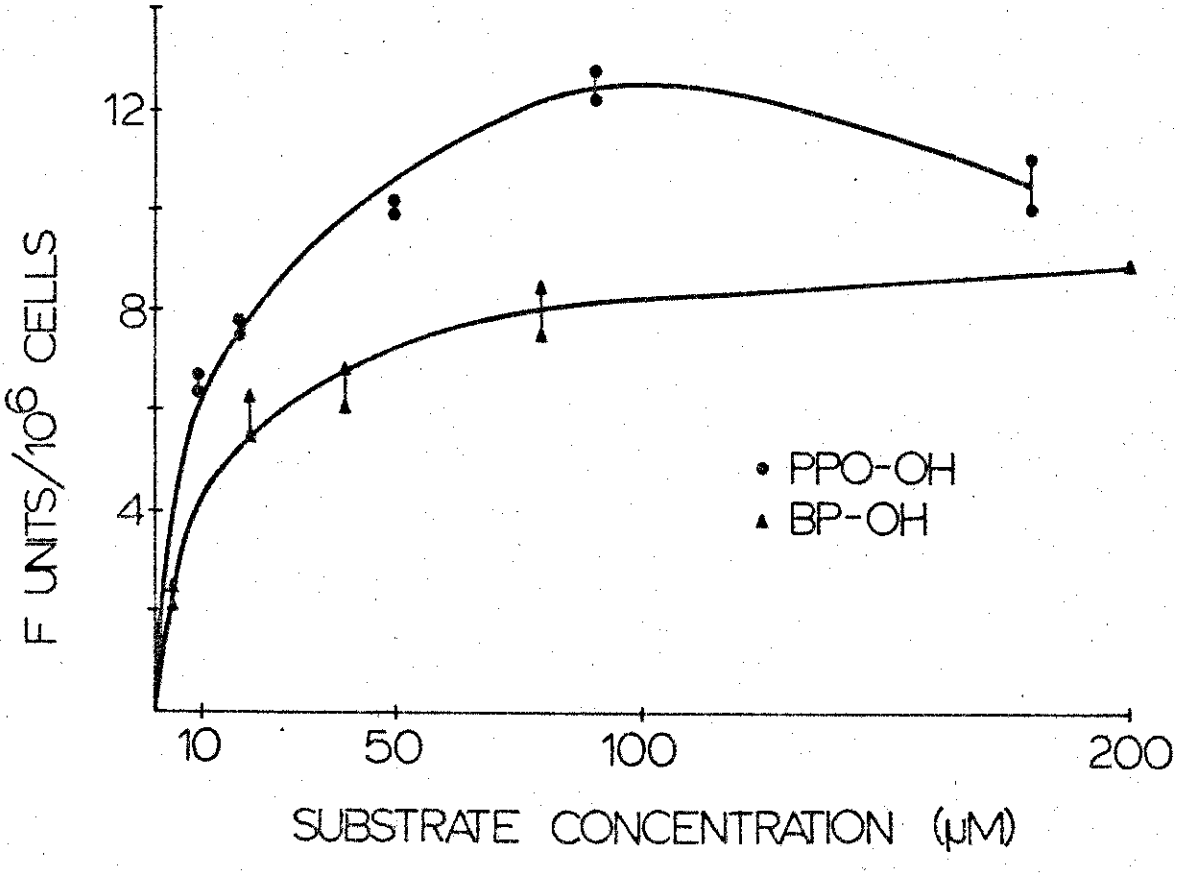

F UNITS/10^6 CELLS
12
8
4
10
50
100
200
SUBSTRATE CONCENTRATION (μM)
PPO-OH
BP-OH

Figure 10. Induction ratios obtained with BP or PPO as substrates in cultured human lymphocytes induced with varying concentrations of BA. Lymphocytes were cultured for 96 hours with no inducer or with varying concentrations of BA. At 96 hours the cells were resuspended in EMSAD buffer (pH 8.0) and incubated at 37°C for 60 minutes with 25 µg BP or 10 µg PPO. The induction ratios were calculated by dividing the BA-induced activity by the activity obtained from the non-induced cells.

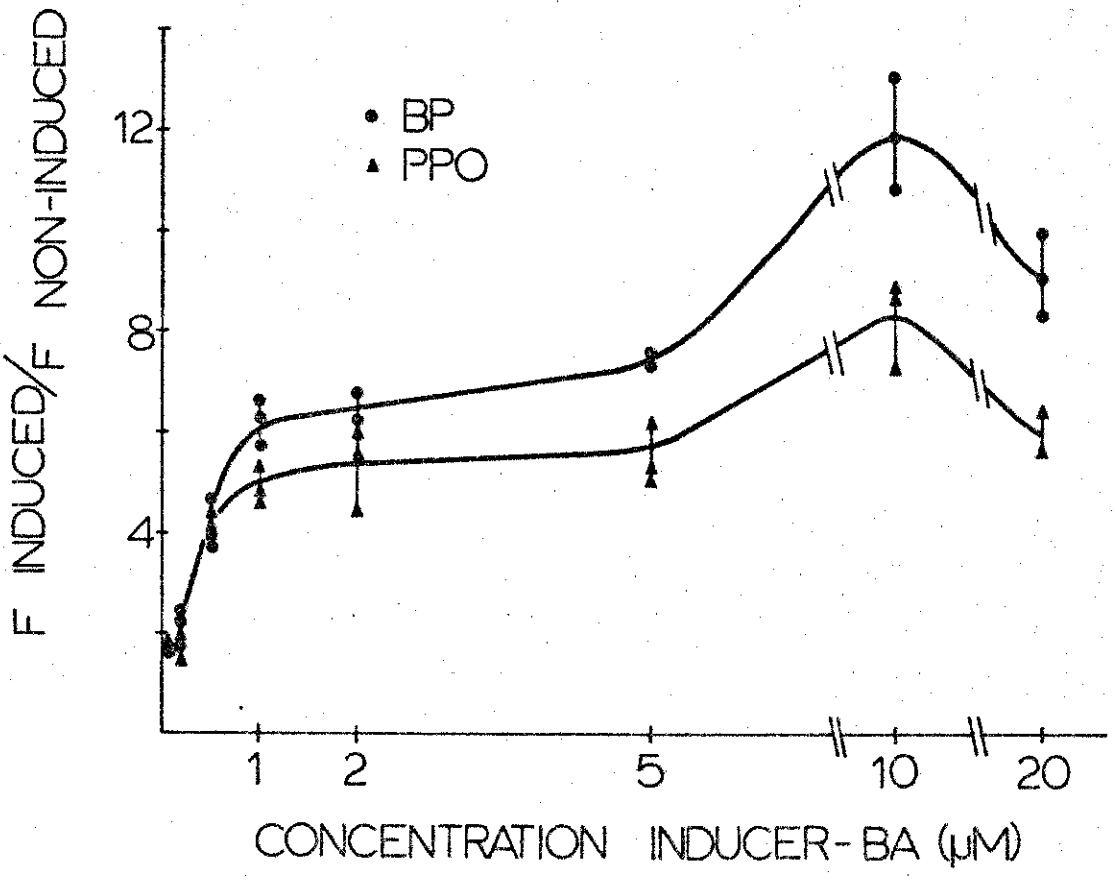

F INDUCED/F NON-INDUCED
12
8
4
BP
PPO
1
2
5
10
20
CONCENTRATION INDUCER-BA (μM)

Figure 11. Effect of concentration of PPO in culture medium on AHH activity in cultured human lymphocytes. PPO was added to duplicate 72-hour lymphocyte cultures in the indicated concentrations. AHH was measured 24 hours later with BP as the substrate.

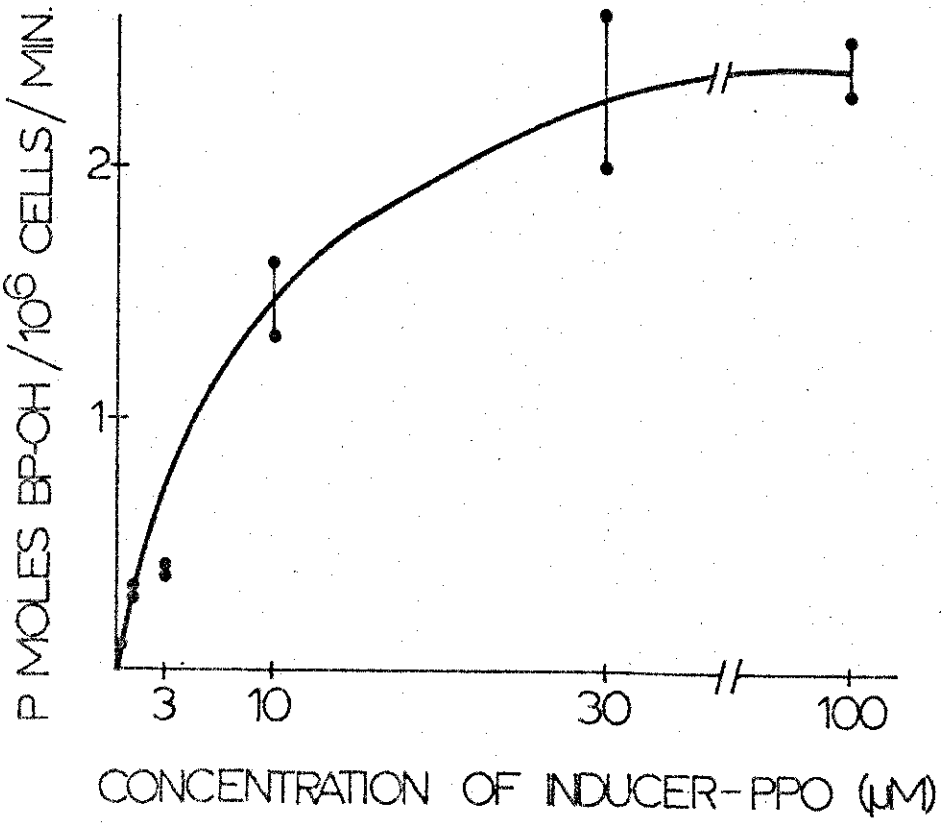

P MOLES BP-OH /10⁶ CELLS / MIN.
2
1
3
10
30
100
CONCENTRATION OF INDUCER-PPO (μM)

Figure 12. Effect of concentration of BA in culture medium on AHH activity in cultured human lymphocytes. BA was added to duplicate 72-hour lymphocyte cultures in the indicated concentrations. AHH activity was measured 24 hours later with BP as the substrate.

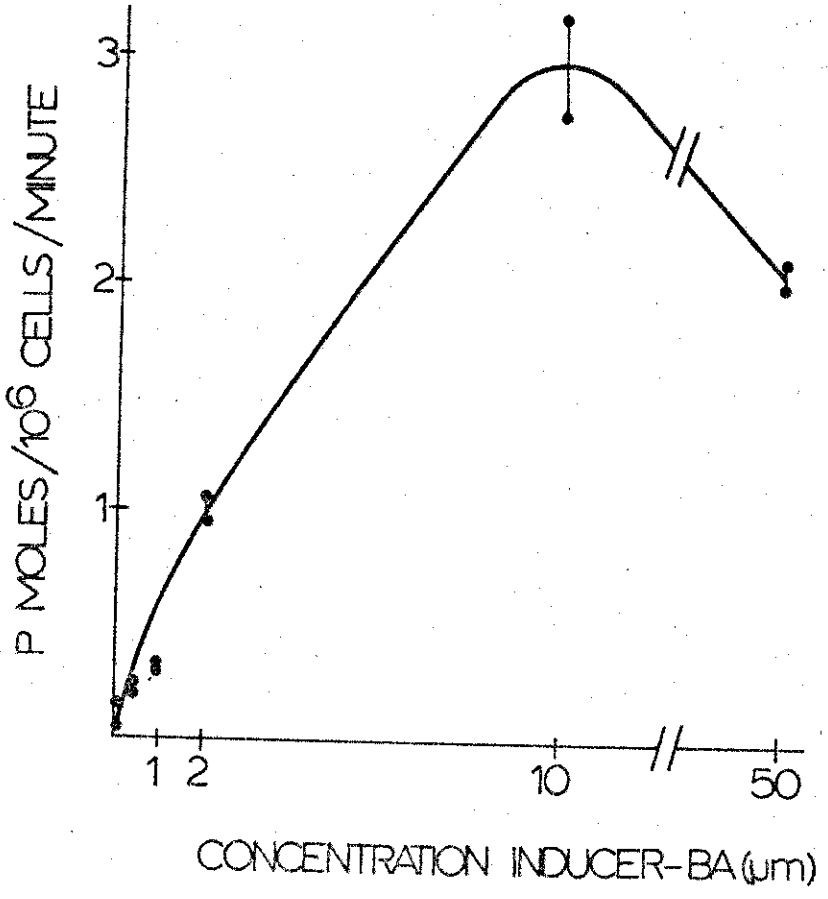

P MOLES/10⁶ CELLS/MINUTE
3
2
1
1 2
10
50
CONCENTRATION INDUCER-BA (µm)

Figure 13. Effect of concentration of MC in culture medium on AHH activity in cultured human lymphocytes. MC was added to duplicate 72-hour lymphocyte cultures in the indicated concentrations. AHH activity was measured 24 hours later with BP as the substrate.

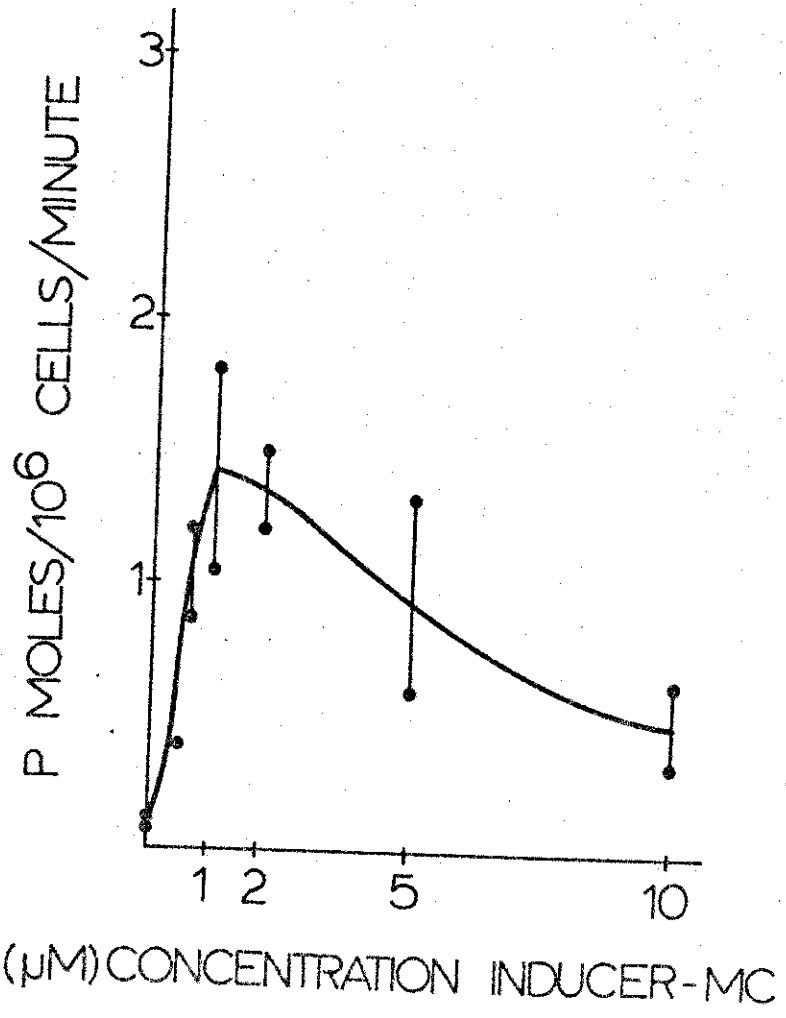

P MOLES/10^6 CELLS/MINUTE
3
2
1
1 2 5 10
(μM) CONCENTRATION INDUCER-MC

Figure 14. The effects of PPO and other known inducers of AHH activity on metabolism of BP and PPO by rat hepatic microsomes. Groups of six Sprague-Dawley rats were injected intraperitoneally on four consecutive days prior to sacrifice. Doses were as follows: MC (20mg/kg), PPO (20 mg/kg), PB (80 mg/kg). Duplicate 1.0 ml aliquots of microsomes containing approximately 100 µg protein were incubated at 37°C for 15 minutes with 1.0 mg NADPH and either 10 µg BP or 10 µg PPO. The vertical lines depict the S.E.M.

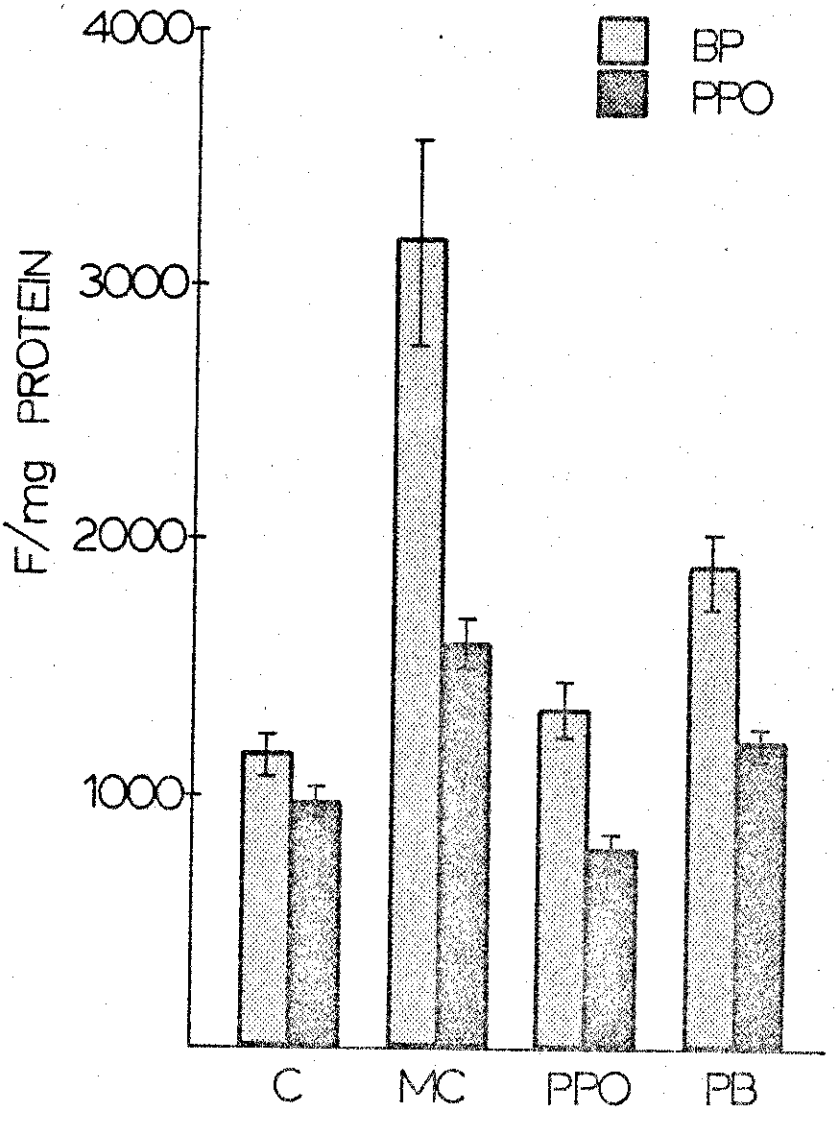

4000
3000
2000
1000
F/mg PROTEIN
BP
PPO
C
MC
PPO
PB

Figure 15. The effects of PPO and other known inducers of AHH activity on metabolism of BP and PPO by immature rat hepatic microsomes. Groups of six immature Sprague-Dawley rats (1-2 months) were injected intraperitoneally on four consecutive days prior to sacrifice. Doses were as follows: MC (20 mg/kg), PPO (20 mg/kg), PPO (200 mg/kg), and PB (80 mg/kg). Duplicate 1.0 ml aliquots of pooled microsomes containing approximately 100 µg protein were incubated at 37°C for 15 minutes with 1.0 mg NADPH and either 10 µg BP or 10 µg PPO.

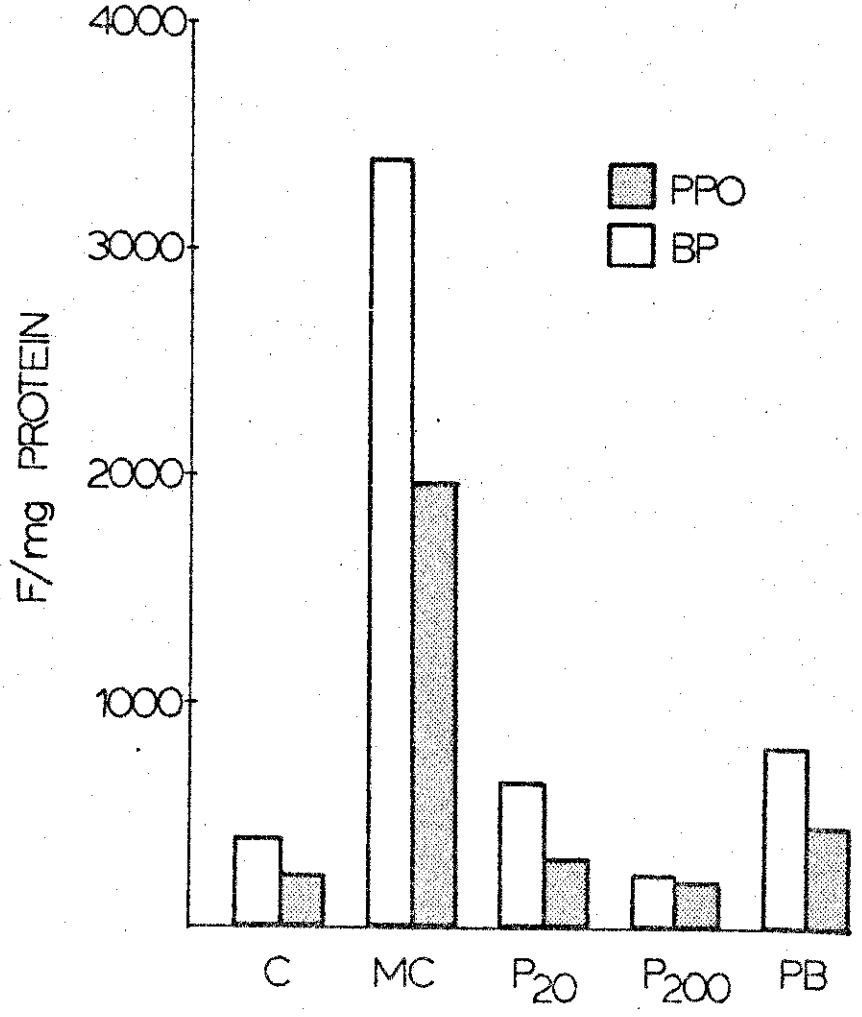

4000
3000
2000
1000
F/mg PROTEIN
PPO
BP
C
MC
P20
P200
PB

Figure 16. The effects of PPO and other known inducers of AHH activity on metabolism of BP and PPO by C57Bl/6J mouse hepatic microsomes. Groups of five C57Bl/6J mice were injected intraperitoneally on four consecutive days with PB (80 mg/kg), BA (50 mg/kg), PPO (50 mg/kg), or PPO (200 mg/kg). Duplicate 1.0 ml aliquots of liver microsomes containing approximately 100 µg protein were incubated at 37°C for 15 minutes with 1.0 mg NADPH and either 10 µg BP or 10 µg PPO. The vertical lines depict the S.E.M.

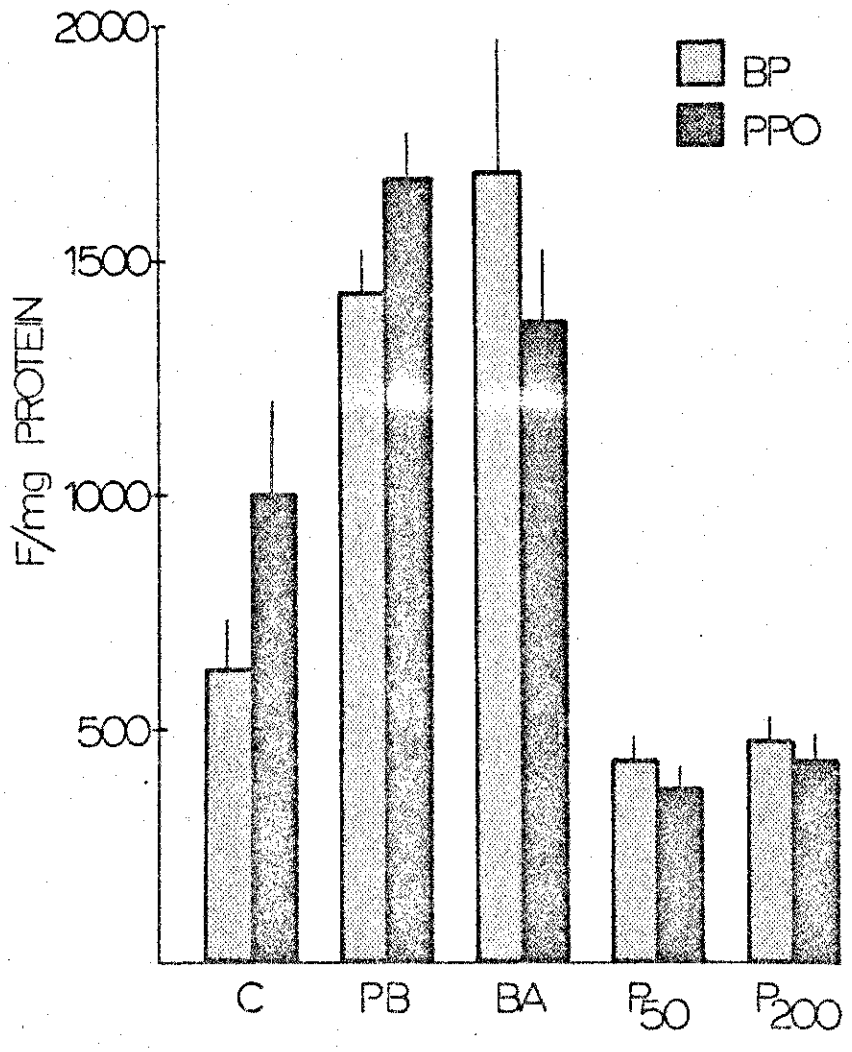

2000
1500
1000
500
F/mg PROTEIN
BP
PPO
C
PB
BA
P50
P200

Table I. Effect of AHH inhibitors on PPO and BP metabolism in cultured human lymphocytes. Human lymphocytes were incubated for 96 hours either without an inducing compound, with 100 μM phenobarbital (PB), or with 10 μM BA. Cells were harvested and resuspended in EMSAD buffer (pH 8.0) at concentration of 5.0 - 7.0 x 10^6 cells/ml. Duplicate 1.0 ml aliquots received ANF (12.5 μg) or SKF-525A (15 μg) and were incubated for 1.0 minute prior to the addition of 10 μg PPO or 10 μg BP. Another set of samples received substrate only (10 μg PPO or 10 μg BP). After incubated 60 minutes at 37°C, the reactions were stopped with 4 ml of 10% acetone in hexane and the organic layer transferred to 1.0 ml of 1N NaOH. Fluorescence was measured for BP-OH with excitation at 396 nm and emission at 522 nm; PPO-OH was measured with excitation at 345 nm and emission at 510 nm.

	CONTROL		+ ANF		+ SKF-525A		
	PPO	BP	PPO	BP	PPO	BP	
NON-INDUCED	1.67	2.52	0.11	0.54	1.16	4.56	$F/10^6$ CELLS
			(7%)	(22%)	(69%)	(155%)	% CONTROL ACTIVITY
PB-INDUCED	2.37	3.81	0.11	0.88	1.32	6.00	$F/10^6$ CELLS
			(5%)	(23%)	(56%)	(157%)	% CONTROL ACTIVITY
BA-INDUCED	15.82	30.45	0.63	6.55	10.92	34.40	$F/10^6$ CELLS
			(4%)	(22%)	(69%)	(113%)	% CONTROL ACTIVITY

Table II. Effect of AHH inhibitors on PPO and BP metabolism in rat liver microsomes. Groups of six Sprague-Dawley rats were injected intraperitoneally on four consecutive days prior to sacrifice and liver microsome preparation. The doses were: MC (20 mg/kg), PB (80 mg/kg), non-induced (0.2 ml corn oil). Duplicate 1.0 ml aliquots of hepatic microsome preparations containing approximately 100 µg protein received 1.0 mg NADPH and the inhibitors and substrates were added as in Table I. Incubations were stopped at 15 minutes and fluorescent measurements determined as indicated in Table I.

	CONTROL		+ ANF		+SKF-525A		
	PPO	BP	PPO	BP	PPO	BP	
NON-INDUCED	356.7	403.3	140.0	413.3	203.3	206.7	F/MG PROTEIN
			(39%)	(102%)	(57%)	(51%)	% CONTROL ACTIVITY
PB-INDUCED	428.8	701.9	163.5	825.0	282.7	544.2	F/MG PROTEIN
			(38%)	(118%)	(66%)	(78%)	% CONTROL ACTIVITY
MC-INDUCED	2185.7	3142.9	502.9	1280.0	1771.4	3228.6	F/MG PROTEIN
			(23%)	(41%)	(81%)	(103%)	% CONTROL ACTIVITY

CHAPTER IV

DISCUSSION

The chemical, 2,5-diphenyloxazole (PPO), is used extensively in laboratories as a primary fluor in liquid scintillation-counting cocktails. It is an effective inducer of AHH in fetal hamster cells (58), mouse prostate cells (54), and rat-derived hepatoma and hepatocyte cell lines (66). The toxicity has been reported as 1.5 g/kg in white rats (42), but there is no information available concerning its carcinogenicity. The apparent low toxicity of PPO, its possible lack of carcinogenicity, and its efficient metabolism by AHH provide some indication for the use of PPO in studies of chemical carcinogenesis and drug metabolism in model systems.

In preliminary experiments in which PPO was employed as an inducer of AHH in cultured human lymphocytes, a fluorescent metabolite of PPO was recovered from the culture medium after the incubation period.

The pH and solvent effects on fluorescence spectral shifts suggested the presence of an alkali-ionizable group such as a phenolic hydroxyl. Additional evidence that the principal metabolite is a phenolic hydroxylated compound are that the chromatographic properties are

similar to those of hydroxybenzo(α)pyrene and the fluorescence color changed after exposure to ammonia vapor on TLC plates. Both of the major metabolites contribute to the fluorescence spectrum of the alkali soluble fraction. Specific identification of the metabolites has not been determined since there is no hydroxylated-PPO standard available for comparison.

In order to partially characterize the metabolism of PPO as it compares with BP metabolism, liver microsomes from 1,2-benzanthracene (BA)-treated and control C57Bl/6J mice were employed for kinetic studies. Kinetic studies of AHH are complicated by a number of factors. The low solubility of BP in aqueous solutions and its propensity for binding to non-hydroxylating sites on proteins have been described by Hansen and Fouts (35). The studies with PPO suggested that the assay conditions as used for BP were also optimal for measuring PPO hydroxylation. The characteristics of PPO metabolism by the microsomal enzyme complex seem to parallel those of BP in several respects. The extent of formation of fluorescent metabolites is within the same order of magnitude, the reaction products may be extracted and assayed in the same manner for both BP and PPO is dependent on NADPH and inhibited by CO. The cumulative data suggest that both reactions are mediated by the AHH system.

Cultured human lymphocytes metabolized PPO to approximately the same extent as BP, but some differences were noted. PPO metabolism was consistently greater than BP metabolism in non-induced lymphocytes, while BP metabolism was greater in PAH-induced lymphocytes. Further differences in PPO and BP metabolism were noted in microsomes from variously induced rats and mice. It is interesting that PPO metabolism was greater than BP metabolism only in the C57Bl/6J control and phenobarbital-treated hepatic microsomes. The C57Bl/6J strain of mice is "genetically responsive" to treatment by AHH inducers and has been used extensively in AHH studies (62).

PPO was more efficiently metabolized than BP by both the constitutive and PB-induced enzymes of C57Bl/6J mice. The variation between PPO and BP metabolism by constitutive and induced enzymes provides evidence for different enzyme binding site(s) in microsomes.

It has been shown that substrate specificity is directly related to the species of cytochrome P-450 present (36). With various substrates the various forms of cytochrome P-450 show preferential hydroxylation at specific positions. The different forms of cytochrome P-450 can be preferentially induced or inhibited by various compounds (27).

An approach to differentiation between types of

cytochrome is to observe the preferential inhibition of metabolism *in vitro* by selected chemicals. The relative amounts of species of cytochrome present in rat hepatic microsomes and cultured human lymphocytes were estimated in this study by employing two inhibitors. For simplicity, the terminology of Wiebel *et al* (83) is adapted here to describe *in vitro* effects of 7,8-benzoflavone (ANF) and SKF-525A upon BP hydroxylation. Hydroxylation of BP *in vitro* by constitutive or PB-induced hepatic microsomes (P-450) was enhanced in the presence of ANF but inhibited in the presence of SKF-525A. Conversely, AHH activity in PAH-induced (P-448) rat hepatic microsomes was inhibited by ANF but enhanced by SKF-525A (83, 29). ANF could not differentiate constitutive and PAH-induced enzyme of extrahepatic tissues, suggesting that extrahepatic AHH activity is "P-448" and not "P-450". Although the ANF effect in control and PAH-induced extrahepatic tissue was not as dramatic as in liver, there was a greater inhibition of PAH-induced enzyme than of constitutive (82, 40).

In the present study, ANF enhanced hydroxylation of BP by constitutive or PB-induced rat hepatic microsomes but inhibited BP hydroxylation by MC-induced hepatic microsomes. These data are in agreement with those reported by Wiebel *et al* (83). In contrast, PPO hydroxylation

was inhibited by ANF in all of the corresponding three groups of rat hepatic microsomes. The effects of SKF-525A on BP hydroxylation were in agreement with those in the literature, although PPO hydroxylation was inhibited in the MC-induced microsomes. ANF and SKF-525A apparently compete at the active site for PPO binding regardless of the type of P-450 present.

In lymphocytes, the pattern of ANF inhibition was the same for BP and PPO metabolism. The metabolism of either substrate was markedly inhibited by ANF in control, PB-, or BA-treated cells. The marked inhibition of BP hydroxylation by ANF in non-induced and PB-induced lymphocytes provides evidence that the cytochrome present in this tissue is not the same P-450 as that found in hepatic tissue. The effects of SKF-525A on BP and PPO metabolism by lymphocytes also suggest heterogeneity of "P-450" between non-induced and BA-treated cells.

PPO concentrations greater than the optimal dose did not result in cytotoxicity or decreased AHH activity. This is in contrast to the pattern of AHH induction by 1,2-benzanthracene (BA) or 3-methylcholanthrene (MC). Cytotoxicity, as evidenced by decreased activity at greater than optimal inducing concentrations and a narrow range of optimal concentrations, were characteristics of those PAH enzyme inducers. The wide range of concentrations over which PPO can elicit maximal or near maximal enzyme

activity provides an advantage over MC or BA induction because the optimal concentration requirement for PPO as an inducer can vary between individuals.

AHH in hepatic microsomes was depressed by PPO pretreatment. BP metabolism was only slightly enhanced in mature rats treated with a relatively low dose (20 mg/kg) of PPO but larger doses of PPO (200 mg/kg) in immature rats caused depressed metabolism of both BP and PPO. These results were surprising in view of previous experiments in which PPO effectively induced AHH of cultured human lymphocytes and other cell cultures (54, 58, 66).

Possible mechanisms explaining the effects of PPO *in vivo* are 1) inhibition of protein synthesis *in vivo*, 2) inhibition of DNA dependent RNA synthesis, 3) inhibition of heme synthesis, 4) perturbation of the steric configuration of microsomal proteins, and 5) increased rate of degradation of proteins. This unusual effect(s) of PPO *in vivo* requires further investigation.

The present data do not support the proposal for use of PPO as an alternate substrate for measuring AHH nor do they support the suggestion to use PPO as an alternate inducer of AHH activity in cultured cells. The results do suggest need for further investigation of PPO as a potential tool for differentiating the cytochromes of the P-450 family.

The variation in BP and PPO metabolism in different tissues probably reflects the variation in types or ratios of different P-450/P-448 linked mixed-function oxygenases (MFO) present and also the relative amounts of hydratase, transferase, and conjugase.

Selective induction or inhibition of these enzyme systems can alter the formation of different BP metabolites. Preferential inhibition or enhancement of enzymes by PPO may provide a tool for elucidating the role of various cytochrome-linked MFO and related detoxification enzymes which may be associated with risk of cancer due to exposure to the polycyclic aromatic hydrocarbons.

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